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Education

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Publications

Review articles and theses:

Tranel, P. J. and **F. Trucco**. 2008. 21st Century Weed Science: A Call for *Amaranthus Genomics*. In N. Stewart (ed.) Genomics of Weedy and Invasive Plants. Blackwell, In Press.

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Fellowships

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Honorary Societies

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Leadership

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THE MOLECULAR BASIS OF DEHYDRATION TOLERANCE IN PLANTS

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ABSTRACT

Molecular studies of drought stress in plants use a variety of strategies and include different species subjected to a wide range of water deficits. Initial research has by necessity been largely descriptive, and relevant genes have been identified either by reference to physiological evidence or by differential screening. A large number of genes with a potential role in drought tolerance have been described, and major themes in the molecular response have been established. Particular areas of importance are sugar metabolism and late-embryogenesis-abundant (LEA) proteins. Studies have begun to examine mechanisms that control the gene expression, and putative regulatory pathways have been established. Recent attempts to understand gene function have utilized transgenic plants. These efforts are of clear agronomic importance.

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INTRODUCTION

This review considers molecular mechanisms involved in dehydration tolerance in plants. Most plants encounter at least transient decreases in relative water content at some stage of their life, and many also produce highly desiccation-tolerant structures such as seeds, spores, or pollen. Indeed, physiological drought also occurs during cold and salt stresses, when the main damage caused to the living cell can be related to water deficit (84, 124). Although we are still far from a complete understanding of the damage caused by drought, or the plant's tolerance mechanisms, much molecular data has been collected over the past few years. Current knowledge of the regulatory network governing the drought-stress responses is also fragmentary, with almost no information on signal perception. However, signal transduction, via ABA at least, and the promoter modules of several response genes, are starting to be elucidated.

Some of the most recent efforts to understand gene function have used transgenic plants, and these studies have significant implications for crop development. Plant breeding has already provided an enormous improvement in the drought tolerance of crop plants (1), with selection often allowing desired traits to be transferred from close wild relatives. However, most of the traits are complex, and their molecular basis is frequently not understood. With our rapidly expanding knowledge of the underlying molecular processes involved in dehydration tolerance, together with the technology of gene manipulation, crop improvement can now also be based on genetic material transferred from any organism and used in a directed manner.

RESEARCH STRATEGIES

Dehydration tolerance has been investigated using three main approaches in plants: (*a*) examining tolerant systems, such as seeds and resurrection plants; (*b*) analyzing mutants from genetic model species; and (*c*) analyzing the effects of stress on agriculturally relevant plants.

Tolerant Systems

One approach of physiological research in dehydration tolerance has been to use specific structures or species that can withstand severe desiccation. Most prominent in this category are certain seeds (73, 82), but desiccation-tolerant species such as resurrection plants (angiosperms) (8), mosses (particularly *Tortula ruralis*), and ferns (98) are also included. Both seeds and the resurrection plant *Craterostigma plantagineum* survive severe dehydration; therefore, the detailed molecular analyses of these systems should reveal expressed genes that contain the genetic information for desiccation tolerance.

SEEDS The final maturation stage of the development of seeds is characterized by desiccation, and as much as 90% of the original water is removed in attaining a state of dormancy with unmeasurable metabolism (73). This desiccated state allows survival under extreme environmental conditions and favors wide dispersal. The embryo cannot withstand desiccation at all developmental stages; tolerance is usually acquired well before maturation drying but is lost as germination progresses. The seeds of many species have been used to isolate the mRNA and proteins related to the desiccation-tolerance response, including, in particular, those of *Arabidopsis thaliana* (100) and of crop species such as cotton (*Gossypium* spp.) (6), barley (*Hordeum vulgare*) (9), maize (*Zea mays*) (99), and rice (*Oryza sativa*) (91). However, a significant complication with these studies is the difficulty of separating the pathways leading to desiccation tolerance from those involved with other aspects of development.

The main achievement of molecular studies with seeds has been the identification and characterization of the late-embryogenesis-abundant (LEA) proteins. LEA-protein mRNAs first appear at the onset of desiccation, dominate the mRNA population in dehydrated tissues (111), and gradually fall several hours after embryos begin to imbibe water (see section on Late-Embryogenesis-Abundant Proteins).

RESURRECTION PLANTS Resurrection plants are unique among angiosperms in their ability to survive during drought, when protoplastic desiccation can leave <2% relative water content in the leaves (8). When water is withheld from mature individuals of *C. plantagineum,* changes rapidly occur at the mRNA and protein levels (8), eventually leading to the tolerant state. A particular advantage of these plants in studies at the molecular level is that desiccation tolerance can be investigated in both whole plants and undifferentiated callus cultures (Tolerant callus of *C. plantagineum* is obtained by pretreatment with ABA) (8). In the callus tissue, and to a certain extent in whole *C. plantagineum* plants, the transition to the tolerant state is largely free of the complications of development or other adjustments inherent in seeds or other plant systems. One of the most

striking features of the desiccation-induced genes characterized from vegetative tissues of *C. plantagineum* has been their similarity to the genes expressed in seeds of other species.

Genetic Model Systems

Genetic model systems are a second major approach to the examination of dehydration tolerance. These systems take advantage of detailed genetic information, a wide range of mutants, and the feasibility of positional gene cloning. Progress in understanding the role of ABA in desiccation tolerance has been achieved by characterizing mutants, such as the ABA-deficient mutants *flacca* (tomato, *Lycopersicon esculentum*) (22) and *droopy* (potato, *Solanum tuberosum*) (108). A number of mutations related to ABA action are also available in *A. thaliana,* and their analysis has provided many insights into ABA-mediated drought responses. *A. thaliana* lines that are less sensitive to ABA than the wild-type have mutations at the *abi* loci [43; see also the maize *vp1* mutant (82)]. The detailed genetic information available for *A. thaliana* facilitated the isolation of the *ABI1* and *ABI3* genes by positional cloning (42, 74, 86). *ABI3* is specifically expressed in seeds and probably encodes a transcription factor able to activate *lea*-type genes (100), and *ABI1* encodes a calcium-regulated phosphatase.

Crop Plants

A third approach in researching dehydration tolerance has been to use species important to agriculture to analyze the plant response after drought stress. This type of study is useful because, through intensive breeding or in vitro selection, lines are available with differing degrees of tolerance. Thus, correlative evidence can be sought for genes putatively involved in the drought response. The transient and moderate drought stress represented in studies of crop species probably describes the most common form of dehydration that most plants are likely to encounter. The intensity of research has thus enabled a much more complete picture of the possible factors involved in drought tolerance to emerge.

GENES WITH UPREGULATED EXPRESSION IN RESPONSE TO DEHYDRATION

To establish the basic responses of plants to drought, two of the approaches already outlined—examination of tolerant systems and crop plants—have been most productive. One type of analysis involves targeting genes thought to be important, such as those for the many enzymes in drought-induced metabolic pathways. A second approach uses differential screening to isolate upregulated genes. These experiments have been successful in describing many genes

encoding proteins of known function associated with desiccation (Table 1). Differential screening has also revealed many genes of unknown function, which are included in Tables 1 and 2; the largest group is the array of LEA-protein-related genes (Table 3). Some of the genes may be involved in secondary problems of drought-stressed plants, such as increased susceptibility to pathogens, e.g. *pcht28* (encoding an acidic endochitinase) (Table 1; 17) and SC514 (encoding lipoxygenase) (Table 1; 10). Genes involved in signaling

Table 1 Genes upregulated by drought stress[a] and encoding polypeptides of known function

cDNA	Source	Encoded polypeptide	Ref
GapC-Crat	*Craterostigma plantagineum*	Cytosolic glyceraldehyde 3-phosphate dehydrogenase	129
pSPS1	*C. plantagineum*	Sucrose-phosphate synthase	[b]
pSS1; pSS2	*C. plantagineum*	Sucrose synthases	36
pPPC1	*Mesembryanthemum crystallinum*	Phosphoenolpyruvate carboxylase	130
pBAD	*Hordeum vulgare* (barley)	Betaine aldehyde dehydrogenase	54
cAtP5CS	*Arabidopsis thaliana*	δ^1-pyrroline-5-carboxylate synthetase	145
RD28	*A. thaliana*	Water channel	141
SAM1; SAM3	*Lycopersicon esculentum* (tomato)	*S*-adenosyl-L-methionine synthetases	37
rd19A; rd21A	*A. thaliana*	Cysteine proteases	67
UBQ1	*A. thaliana*	Ubiquitin extension protein	66
pMBM1	*Triticum aestivum* (wheat)	L-isoaspartyl methyltransferase	90
SC514	*Glycine max* (soybean)	Lipoxygenase	10
cATCDPK1; cATCDPK2	*A. thaliana*	Ca^{2+}-dependent, calmodulin-independent protein kinases	127
PKABA1	*T. aestivum*	Protein kinase	4
cAtPLC1	*A. thaliana*	Phosphatidylinositol-specific phospholipase C	53
Apx1 gene	*Pisum sativum* (pea)	Cytosolic ascorbate peroxidase	89
Sod 2 gene	*P. sativum*	Cytosolic copper/zinc superoxide dismutase	135
P31	*L. esculentum*	Cytosolic copper/zinc superoxide dismutase	102
pcht28	*L. chilense*	Acidic endochitinase	17
Atmyb2	*A. thaliana*	MYB-protein-related transcription factor	128
ERD11; ERD13	*A. thaliana*	Glutathione *S*-transferases	63
cAtsEH	*A. thaliana*	Soluble epoxide hydrolase	61

[a]The best-characterized plant genes from which cDNA clones have been demonstrated to show increased mRNA expression levels in response to drought stress have been included. Drought stress has been taken to include quite diverse treatments, ideally where water has been withheld from the plant, but also for example by applying osmotic stress with mannitol solutions or by detaching plant organs.

[b]Ingram & Bartels, unpublished data.

and control processes are considered in the section on Second Messengers and Signaling Molecules.

Metabolism

Changes in primary metabolism are a general response to stress in plants. For example, a cDNA-encoding glyceraldehyde-3-phosphate dehydrogenase, isolated from the resurrection plant *C. plantagineum* (Table 1; 129), shows increased expression during drought and upon ABA treatment. However, increased levels of the enzyme are also associated with other environmental stresses in plants, possibly reflecting increased energy demand. Proteases may also be an important feature of stress metabolism, dispensing with redundant proteins and depolymerizing vacuolar storage polypeptides, thereby releasing amino acids for the massive synthesis of new proteins (Tables 1 and 2; 50).

Enzymes of sugar metabolism are probably critical in desiccation tolerance. It has been demonstrated that certain sugars may be central to the protection of a wide range of organisms against drought (see section on Sugars). In *C. plantagineum,* the overall transcript levels of sucrose-phosphate synthase and sucrose synthase increase immediately in response to drought (36; J Ingram & D Bartels, unpublished data). The expression pattern is complex if the kinetics of individual transcript types are followed over the entire course of dehydration.

Enzymes involved in the synthesis of other compounds that can act as compatible solutes—and whose transcript levels are clearly upregulated during drought—include $\delta\Delta^1$-pyrroline-5-carboxylate synthetase (proline biosynthesis) (Table 1; 145) and betaine aldehyde dehydrogenase (glycine betaine biosynthesis) (Table 1; 54).

The induction of the mRNA encoding phospho*enol*pyruvate carboxylase in *Mesembryanthemum crystallinum* (Table 1; 130) highlights the importance of Crassulacean acid metabolism in enabling carbon fixation with minimal water loss. Such metabolism is a major response in a wide variety of plants to growth in dry conditions (139).

Osmotic Adjustment

Total water potential can be maintained during mild drought by osmotic adjustment, which involves utilizing sugars or other compatible solutes (12). Both ion and water channels are likely to be important in regulating water flux, and the relevance of these channels to drought-stress has been supported by the isolation of channel protein genes expressed in response to water deficit. The 7a cDNA from pea (*Pisum sativum*) (Table 2; 50) encodes a polypeptide with characteristic features of ion channels, while the RD28 cDNA (*A. thaliana*) (Table 1; 141) and probably also the H2-5 cDNA (*C. plantagineum*)

Table 2 Genes upregulated by drought stress[a] but encoding polypeptides of unknown function

cDNA	Source	Features of encoded polypeptide	Ref
26g	*Pisum sativum* (pea)	Some similarity to aldehyde dehydrogenase	50
7a	*P. sativum*	Similar to channel proteins	50
kin2	*Arabidopsis thaliana*	Similarity to animal antifreeze proteins	69
pcC 37-31	*Craterostigma plantagineum*	Similar to early-light-inducible proteins	7
TSW12	*Lycopersicon esculentum* (tomato)	A lipid transfer protein	125
pLE16	*L. esculentum*	Similar to lipid transfer proteins	107
15a	*P. sativum*	Similarity to proteases	50
pA1494	*A. thaliana*	Similarity to proteases	136
ERD1	*A. thaliana*	Similar to a Clp ATP-dependent protease subunit	64
Ha hsp17.6; Ha hsp17.9	*Helianthus annuus* (sunflower)	Low-molecular-weight heat-shock proteins	21
Athsp70-1	*A. thaliana*	Similar to the HSP70 heat-shock-protein family	66
Athsp81-2	*A. thaliana*	Similar to the HSP81 heat-shock-protein family	66
BLT4	*Hordeum vulgare* (barley)	Similar to protease inhibitors	32
P22	*Raphanus sativus* (radish)	Similar to protease inhibitors	77
BnD22	*Brassica napus* (rape)	Similar to protease inhibitors	31
pMAH9	*Zea mays* (maize)	Similar to RNA-binding proteins	47
MsaciA	*Medicago sativa* (alfalfa)	Similar to pUM90-1 and pSM2075 polypeptides	70
pUM90-1	*M. sativa*	Similar to MsaciA and pSM2075 polypeptides	80
pSM2075	*M. sativa*	Similar to MsaciA and pUM90-1 polypeptides	79
pBN115	*B. napus*	Similar to polypeptides encoded by pBN19 and pBN26 (*B. napus*), and COR15 (*A. thaliana*)	134
RD22	*A. thaliana*	Similar to an unidentified seed protein from *Vicia faba*	56
salT	*Oryza sativa* (rice)		18
lti65 gene; *lti78* gene	*A. thaliana*		95
pcC 13-62	*C. plantagineum*		104

[a]See Footnote a in Table 1.

(J-B Mariaux & D Bartels, unpublished data) encode putative water-channel proteins (28).

Structural Adjustment

Drought stress has been shown to cause alterations in the chemical composition and physical properties of the cell wall (e.g. wall extensibility), and such changes may involve the genes encoding *S*-adenosylmethionine synthetase (Table 1; 37). Under nonstressful conditions, increased expression of *S*-adenosyl-L-methionine synthetase genes correlates with areas where lignification is occurring (101). Thus, the increased expression in drought-stressed tissue could thus also be due to lignification in the cell wall. Cell elongation stops under prolonged drought stress, and then lignification processes seem to begin (94a). Espartero et al (37) also noted that fungal elicitors cause the coinduction of *S*-adenosyl-L-methionine synthetase transcript with those of other enzymes, e.g. *S*-adenosyl-L-homocystein hydrolase or a methyltransferase, required for cell wall formation.

The *C. plantagineum pcC37-31* cDNA (Table 2; 7) encodes the dsp-22 protein, whose mRNA levels increase in response to various stresses. The cDNA shows significant homology to early light-inducible protein (ELIP) genes (1a). Light is involved in the regulation of the gene expression, and the encoded dsp-22 protein is chloroplastic. ELIPs may play a role in the assembly of the photosystem (1a). During desiccation, *C. plantagineum* chloroplasts undergo morphological changes, and thus the dsp-22 protein could bind pigments or help maintain assembled photosynthetic structures essential for resuming active photosynthesis during resurrection.

Degradation and Repair

Genes encoding proteins with sequence similarity to proteases, and which are induced by drought, have been isolated from both pea (Table 2; 50) and *A. thaliana* (Tables 1 and 2; 64, 67, 136). One of the functions of these enzymes could be to degrade proteins irreparably damaged by the effects of drought (50). During early drought in *A. thaliana,* there is an increase in levels of mRNA encoding ubiquitin extension protein (66), a fusion protein from which active ubiquitin is derived by proteolytic processing. This increase may be significant in terms of protein degradation, because ubiquitin has a role in tagging proteins for destruction. During drought stress, protein residues may be modified by chemical processes such as deamination, isomerization, or oxidation, and it is thus likely that enzymes with functions in protein repair are upregulated in response to drought. Indeed, the response to desiccation in mosses may largely be repair based (98). An example of such repair processes is the observation that L-isoaspartyl methyltransferases may convert modified L-isoaspartyl residues in damaged proteins back to L-aspartyl residues (Table 1; 90).

Mudgett & Clarke (90) have argued that such repair mechanisms could be particularly important during desiccation, when protein turnover rates are low. Although *Escherichia coli* mutants lacking the enzyme grow normally in the logarithmic phase when there is high protein turnover, they survive poorly in the stationary phase when turnover is much lower (75).

The products of two drought-induced genes isolated by differential screening have sequence similarity to heat-shock proteins (Table 2; 66). These encoded proteins are probably chaperonins, involved in protein repair by helping other proteins to recover their native conformation after denaturation or misfolding during water stress. The low-molecular-weight heat-shock proteins (Table 2; 21) may also be chaperonins. This function has been demonstrated for a mammalian low-molecular-weight heat-shock protein (58). An alternative function may be in the sequestration of specific mRNAs in cells subjected to drought (96).

Removal of Toxins

Enzymes concerned with removing toxic intermediates produced during oxygenic metabolism, such as glutathione reductase and superoxide dismutase, increase in response to drought stress and are probably very important in tolerance (89). Decreasing leaf water content and consequent stomatal closure result in reduced CO_2 availability and the production of active oxygen species such as superoxide radicals (117). Increased photorespiratory activity during drought is also accompanied by elevated levels of glycolate-oxidase activity, resulting in H_2O_2 production (89). This could explain why genes encoding enzymes that detoxify active oxygen species such as ascorbate peroxidase (Table 1; 89) and superoxide dismutase (Table 1; 102, 135) have been found upregulated in response to drought.

Late-Embryogenesis-Abundant Proteins

The genes encoding late-embryogenesis-abundant (LEA) proteins are consistently represented in differential screens for transcripts with increased levels during drought. LEA proteins were first described from research into genes abundantly expressed during the final desiccation stage of seed development (see above). Circumstantial evidence for their involvement in dehydration tolerance is strong: The genes are similar to many of those expressed in vegetative tissues of drought-stressed plants (Table 3), and desiccation treatments can often induce precocious expression in seeds. ABA can also induce the *lea* genes in seeds and vegetative tissues.

GENERAL FEATURES Groupings for dividing the LEA proteins originate from a dot matrix analysis with proteins from cotton. A group was assigned on the basis of one cotton LEA protein showing regions of significant homology with

Table 3 Genes upregulated by drought stress[a] that encode polypeptides related to late-embryogenesis-abundant LEA proteins

cDNA	Source	Relationship of encoded polypeptide to LEA proteins	Ref
Ha ds10	*Helianthus annuus* (sunflower)	D19-LEA-protein related	3
Em	*Triticum aestivum* (wheat)	D19-LEA-protein related	76
B19.1; B19.3; B19.4	*Hordeum vulgare* (barley)	D19-LEA-protein related	39
pLE25	*Lycopersicon esculentum* (tomato)	D113-LEA-protein related	23
Ha ds11	*H. annuus*	D113-LEA-protein related	3
pRABAT1	*Arabidopsis thaliana*	D11-LEA-protein related	72
pcC 27-04	*Craterostigma plantagineum*	D11-LEA-protein related	104
M3 (RAB-17)	*Zea mays* (maize)	D11-LEA-protein related	20
B8; B9; B17	*H. vulgare*	D11-LEA-protein related	20
pLE4	*L. esculentum*	D11-LEA-protein related	23
pcC 6-19	*C. plantagineum*	D11-LEA-protein related	104
TAS14	*L. esculentum*	D11-LEA-protein related	46
pLC30-15	*L. chilense*	D11-LEA-protein related	16
H26	*Stellaria longipes*	D11-LEA-protein related	110
pRAB 16A	*Oryza sativa* (rice)	D11-LEA-protein related	91
pcECP40	*Daucus carota* (carrot)	D11-LEA-protein related	62
ERD10; ERD14	*A. thaliana*	D11-LEA-protein related	65
pMA2005	*T. aestivum*	D7-LEA-protein related	26
pMA1949	*T. aestivum*	D7-LEA-protein related	27
pcC 3-06	*C. plantagineum*	D7-LEA-protein related	104
pcC 27-45	*C. plantagineum*	D95-LEA-protein-related	104

[a]See Footnote a in Table 1.

at least one protein from another species (33). The "type" of cotton proteins used for these groupings were LEA D19 (Group 1), LEA D11 [Group 2 (also termed dehydrins)], and LEA D7 (Group 3). The cotton proteins LEA D113 (34, 35) and LEA D95 (40) now define two additional classes. This system will remain useful until clear functions can be assigned.

LEA proteins appear to be located in many cell types and at variable concentrations (19, 34, 35, 45), and within the cell they appear to be predominantly—but not exclusively—cytosolic (19, 45, 91, 114). The concentrations in the cell are characteristically very high. For example, in mature cotton embryo cells, the D7 LEA proteins represent about 4% of nonorganellar cytosolic protein (about 0.34 mM) (111).

A general structural feature of the LEA proteins is their biased amino acid composition, which results in highly hydrophilic polypeptides, with just a few residues providing 20–30% of their total complement. For example, a deduced

D19 protein from cotton contains 13% glycine and 11% glutamic acid (6). Furthermore, most LEA proteins lack cysteine and tryptophan residues.

ROLES We await direct experimental evidence that LEA proteins can protect specific cellular structures or ameliorate the effects of drought stress. Because they are highly hydrophilic, it appears unlikely that they occur in specific cellular structures. Also, their high concentrations in the cell and biased amino acid compositions suggest that they do not function as enzymes (6).

The randomly coiled moieties of some LEA proteins are consistent with a role in binding water. Total desiccation is probably lethal, and therefore such proteins could help maintain the minimum cellular water requirement. McCubbin & Kay (83) have found that the Em protein (D19-group) (Table 3; 76) from wheat is considerably more hydrated than most globular polypeptides because it is over 70% random coil in normal physiological conditions. The random coil tails of the D113 proteins could also bind considerable amounts of water, although the long *N*-terminal helical domain would not share this property (34, 35).

A major problem under severe dehydration is that the loss of water leads to crystallization of cellular components, which in consequence damages cellular structures. This may be counteracted by LEA proteins, and some of the LEA proteins could essentially be considered compatible solutes, which supports the likely role of sugars in maintaining the structure of the cytoplasm in the absence of water. Baker et al (6) have suggested that LEA proteins D11 and D113 could be involved in the "solvation" of cytosolic structures. The random coiling would permit their shape to conform to that of other structures and provide a cohesive layer with possibly greater stability than would be formed by sugars. Their hydroxylated groups would solvate structural surfaces. Furthermore, they could be superior to sucrose as protectants in being less likely to crystallize. However, for the D11-related protein RAB-17, a regulatory role has been postulated (see below).

Baker et al (6) have hypothesized that the 11-amino-acid motif (T/A A/T Q/E A/T A/T K/R Q/ED K/R A/T X ED/Q) (34) of LEA protein D-29 (which is also present in D7 LEA proteins) could counteract the irreversibly damaging effects of increasing ionic strength in the cytosol during desiccation. Such problems could be mitigated by the formation of salt bridges with amino acid residues of highly charged proteins. The repeating elements most likely exist as amphiphilic helices (34), which means that hydrophobic and hydrophilic amino acids are contained in particular sectors of the helix. The helices probably form intramolecular bundles, which would present a surface capable of binding both anions and cations. Further analyses of the D7-group molecules have allowed precise structural predictions to be made: The intersurface edges

of the interacting helical regions of the (putative) dimer reveal periodically spaced binding sites for suitably charged ions.

SUGARS

The involvement of soluble sugars in desiccation tolerance in plants is suggested by studies in which the presence of particular soluble sugars can be correlated with the acquisition of desiccation tolerance (73). Such studies have followed work with animals, fungi, yeast, and bacteria, in which a high level of the disaccharide trehalose has been established as important in surviving desiccation. Trehalose is the most effective osmoprotectant sugar in terms of minimum concentration required (25). Whereas trehalose is extremely rare in plants, sucrose—together with other sugars—appears able to substitute. Although sugar accumulation is not the only way in which plants deal with desiccation (12), it is considered an important factor in tolerance.

Many studies with seeds have demonstrated the accumulation of soluble sugars during the acquisition of desiccation tolerance (73); similar results have been demonstrated in resurrection plants. A common theme has emerged. Various soluble carbohydrates may be present in fully hydrated tissues, but sucrose usually accumulates in the dried state. For example, desiccation in the leaves of *C. plantagineum* is accompanied by conversion of the C8-sugar 2-octulose (90% of the total sugar in hydrated leaves) into sucrose, which then comprises about 40% of the dry weight (11).

Total water potential can be maintained during mild drought by osmotic adjustment. Sugars may serve as compatible solutes permitting such osmotic adjustment, although many other compounds usually associated with salt stress are also active, such as proline, glycine betaine, and pinitol (54, 84, 145). Increasing sucrose synthesis and sucrose-phosphate synthase activity is not only a drought-response of desiccation-tolerant plants such as *C. plantagineum* (36) but also of plants that cannot withstand extreme drying, such as spinach (109).

One way sugars may protect the cell during severe desiccation is by glass formation: Rather than solutes crystallizing, through the presence of sugars a supersaturated liquid is produced with the mechanical properties of a solid (68). Glass formation has been demonstrated in viable maize seeds and has been associated with their viability (137). Differential scanning calorimetry has been used to examine the effect of termperature on glass formation by sugar mixtures; only sugar mixtures equivalent in concentration and composition to those in desiccation-tolerant embryos are able to form glass at ambient temperatures (68). It seems likely that sugar composition, rather than just concentration, is related to glass formation. During desiccation, glass would fill space, thus preventing cellular collapse, and in restricting the molecular

diffusion required by chemical reactions would permit a stable quiescent state (68).

Phosphofructokinase is a tetrameric enzyme that usually dissociates irreversibly into inactive dimers during dehydration (14). However, it was found that in vitro the disaccharides sucrose, maltose, and trehalose stabilize the activity of the enzyme during drying.

Crowe et al (24) have shown that, in vitro, drying and rehydration of the model-membrane sarcoplasmic reticulum usually results in the fusion of vesicles and loss of the ability to transport calcium. However, when the sugar trehalose was present at concentrations equivalent to those in desiccation-tolerant organisms, functional vesicles were preserved. Many other studies show that sugars can protect membranes in vitro (25); it is suggested that sugars alter physical properties of dry membranes so that they resemble those of fully hydrated biomolecules.

The mechanism by which proteins are stabilized by sugars is better understood than the situation with membranes. Infrared spectroscopy has shown that trehalose probably forms hydrogen bonds between its hydroxyl groups and polar residues in proteins (25). Hydrogen bonding between the hydroxyl group of trehalose and the phosphate head group of phospholipids can be inferred from comparisons of changes in the infrared spectrum of the molecules during dehydration. Strauss & Hauser (120) used the cation Eu^{3+}, which is known to form a specific ionic bridge to the phosphate of phospholipids, to show that sucrose is probably bound between phosphate sites in dry membranes. This was inferred from experiments in which Eu^{3+} ions were added to preparations of sucrose and phosphatidylcholine vesicles; the stabilization of liposomes by sucrose during freeze drying decreased as the Eu^{3+} ions were added, which suggests competitive binding of sucrose and Eu^{3+} at the phosphate sites of the phospholipids.

REGULATION OF GENE EXPRESSION DURING DEHYDRATION

The machinery leading to the expression of drought-stress genes conforms to the general cellular model, with a complex signal transduction cascade that can be divided into the following basic steps: (*a*) perception of stimulus; (*b*) processing, including amplification and integration of the signal; and (*c*) a response reaction in the form of de novo gene expression. No molecular data are available on the perception of drought stress, although turgor change has been suggested as a possible physical signal. An attractive model for the activation of a transduction pathway by a stress signal has been derived from studying the heat-shock response in yeast (60). Kamada et al (60) suggest that heat-induced activation of a particular pathway is in response to increased

membrane fluidity in the cell wall. The cell detects this weakness in the cell wall by sensing stretch in the plasma membrane. Examples such as this from simple systems may provide the conceptual framework for devising experiments in plants.

The drought-activated signal transmission process has begun to be dissected at the molecular level, mostly on the basis of studies of isolated drought-responsive genes. Endogenous ABA levels have been reported to increase as a result of water deficit in many physiological studies, and therefore ABA is thought to be involved in the signal transduction (15, 43). Many of the drought-related genes can be induced by exogenous ABA; however, this does not necessarily imply that all these genes are also regulated by ABA in vivo.

We now discuss promoter studies, signaling molecules, and both posttranscriptional and posttranslational modifications in the context of drought-regulated gene expression.

Promoter Studies

CIS- AND *TRANS*-ACTING ELEMENTS Many of the changes in mRNA levels observed during drought reflect transcriptional activation. Treatment with ABA can also induce these changes, and this treatment has been utilized for setting up experimental systems to define *cis-* and *trans*-acting elements. *cis-* and *trans*-acting elements involved in ABA-induced gene expression have been analyzed extensively (Tables 4 and 5; 43).

Table 4 *cis*-acting promoter elements relevant to ABA or drought

Gene	Element	Sequence[a]	Ref
Rab16A (*Oryza sativa*)	ABRE (Motif I)	GT*ACGT*GGCGC	119
EM (*Triticum aestivum*)	Em1A	GGAC*ACGT*GGC	51
Hex3 (synthetic tetramer) (derived from *Nicotiana tabacum*)		GGTGACGTGGC	71
rab28 (*Zea mays*)	ABRE	CC*ACGT*GG	106
Cat1 (*Zea mays*)		CCAAGAAGTC-C*ACGT*GGAGGTGGAAGAG	138
HVA22 (*Hordeum vulgare*)	ABRE3 and CE1	GCC*ACGT*ACA and TGCCACCGG	118
CDeT27-45 (*Craterostigma plantagineum*)		AAGCCCAAATTTCA-CAGCCCGATAACCG	93
rd29 (*Arabidopsis thaliana*)	DRE	TACCGACAT	144

[a]The G-box core elements ACGT are in italic.

The best-characterized *cis*-element in the context of drought stress is the ABA-responsive element (ABRE), which contains the palindromic motif CACGTG with the G-box ACGT core element (44). ACGT elements have been observed in a multitude of plant genes regulated by diverse environmental and physiological factors. Systematic DNA-binding studies have shown that nucleotides flanking the ACGT core specify the DNA-protein interactions and subsequent gene activation (57). G-box-related ABREs have been observed in many ABA-responsive genes, although their functions have not always been proven experimentally. The best-studied examples of these ABRE promoter elements are Em1a from wheat and Motif I from the rice *rab 16A* gene (Table 4; 81, 92). Multiple copies of the elements fused to a minimal 35S promoter confer an ABA response to a reporter gene (51, 119), which supports the hypothesis that ABREs are critical for the ABA induction of relevant genes (although it is difficult to explain why single copies are not

Table 5 Characterization of promoters in transgenic plants

Gene	Native gene activity	Reporter gene activity	Ref
Rab 16B	Embryos of Oryza sativa	*Nicotiana tabacum* embryos	142
Em	Embryos of Triticum aestivum	*Nicotiana tabacum* embryos	81
Rab 17	Embryos of Zea mays	The embryos and endosperm of Arabidopsis thaliana	131
Hex3 (synthetic tetramer) (derived from *Nicotiana tabacum*)		Mature seeds of *N. tabacum;* inducible in seedlings by desiccation, salt, and ABA	71
Rd 22	Dehydrated A. thaliana plants	Constitutive in flowers and stems of *A. thaliana;* inducible in *N. tabacum* by ABA or dehydration	56
Rd 29A	Dehydrated A. thaliana plants	Inducible by dehydration in most vegetative parts of *A. thaliana;* inducible in *N. tabacum* by cold, ABA, and salt	143, 144
CDeT27-45	C. plantagineum dehydrated or ABA-treated vegetative tissues	In embryos and mature pollen of both *A. thaliana* and *N. tabacum*	39a, 88
CDeT6-19	C. plantagineum dehydrated or ABA-treated vegetative tissues	In developing embryos and mature pollen of both *A. thaliana* and *N. tabacum* also inducible in their leaves and guard cells	87, 39a, 123
CDeT11-24	C. plantagineum dehydrated or ABA-treated vegetative tissues	Embryos of both *A. thaliana* and *N. tabacum;* inducible in *A. thaliana* leaves by dehydration	[a]
DC8	Embryos of *Daucus carota*	D. carota seed tissues	49
DC3	Embryos of Daucus carota	N. tabacum seedlings; also inducible in the leaves by either drying or ABA treatment	132

[a]R Velasco, F Salamini & D Bartels, unpublished data.

sufficient for this response). The ABA effect on transcription was orientation independent in both the wheat and rice elements, which suggests that they possibly function as enhancer elements in their native genes. Electrophoretic-mobility-shift assays and methylation-interference footprinting have shown that both Em1a and Motif1 interact with nuclear proteins; these DNA-binding proteins are constitutively expressed in an ABA-independent manner (51, 92). cDNAs encoding ABRE-binding proteins (wheat EMBP-1 and tobacco TAF-1) have been cloned and shown to contain a basic region adjacent to a leucine-zipper motif that is characteristic of transcription factors (51, 97). Despite the fact that both proteins exhibit specific and distinct binding properties, their roles in vivo are not understood. It seems possible that they are not directly involved in ABA-responsive gene expression but that they cooperate with other regulatory factors.

Recently, two different elements have been described that must be present to allow a single copy of the ABRE to mediate transcriptional activation in response to ABA, and thus define an ABA response complex. An ABRE element in the barley *Amy32b* α-amylase promoter has been shown to allow ABA-stimulated transcription to increase only in the presence of an O2S element that interacts with the ABRE within tight positional constraints. A second coupling element has been identified during promoter analysis of the ABA-induced barley *HVA22* promoter (118). The coupling element (CE1) acts together with a G-box-type ABRE (GCCACGTACA) in conferring high ABA induction, whereas the ABRE alone is not sufficient for transcriptional activation. CE1-like elements have been found in many other ABA-regulated promoters, but their function remains to be demonstrated (118). The specific sequence of a coupling element may profoundly affect the specificity of ABA-driven gene expression and may explain differences between functional and nonfunctional ABREs.

In promoters such as *CDeT27–45* or *CDeT6–19,* isolated from *C. plantagineum,* G-box-related ABREs do not appear to be major determinants of the ABA or drought response (87, 88). The *CDeT27–45* promoter contains an element that specifically binds nuclear proteins from ABA-treated tissue; this promoter fragment is essential but not sufficient for conferring a response to ABA on a reporter gene (93).

Besides the ABA-mediated gene expression, the investigation of drought-induced genes in *A. thaliana* has also revealed ABA-independent signal transduction pathways (144). The *A. thaliana* genes *rd29A* and *rd29B* are differentially induced under conditions of dehydration, salt or cold stress, and ABA treatment. The *rd29A* gene has at least two *cis*-acting elements. 1. The 9-bp direct repeat sequence, TACCGACAT, termed the dehydration-responsive element (DRE), functions in the initial rapid response of *rd29A* to drought, salt, or low temperature (144). 2. The slower ABA response is medi-

ated by another fragment that contains an ABRE (143). It will be interesting to see whether the same *cis* elements function in other *A. thaliana* genes that are induced during progressive drought; besides ABA, at least two other different signals are involved in this induction (48). The existence of ABA-dependent and -independent pathways is corroborated by studies on the accumulation of three distinct *Lea* transcripts in barley embryos. Selected transcripts increased in response to osmotic stress without requiring ABA, whereas induction by salt did require ABA (38).

A different class of potential transcription factors with relevance to drought stress is represented by the *A. thaliana* gene *Atmyb2*. This gene encodes an MYB-related protein and is induced by dehydration or salt stress and by ABA (128). Plant *myb*-related genes comprise a large family that may play various roles in gene regulation. The ATMYB2 protein expressed in *E. coli* has been shown to bind the MYB-recognition sequence, PyAACTG, which supports its role as a DNA-binding protein. Another *A. thaliana* drought stress–induced gene, *rd22* (56), has a promoter with no ABRE but with two recognition sites for the transcription factors MYC and MYB. Binding of the ATMYB2 protein appears likely but has not been proven experimentally.

ASSESSMENT OF PROMOTERS IN TRANSGENIC PLANTS Promoter analysis using transient expression assays has resulted in the characterization of several distinct *cis*-acting elements and the cloning of related transcription factors. However, tests with a range of promoters derived from drought- or ABA-inducible structural genes in transgenic plants have shown that the promoter activities defined in transient assays are not always correlated with the expression patterns of their corresponding structural genes. A summary of results is given in Table 5. A problem with the approach could be the use of heterologous plant expression systems. Although the genes are always active in seeds, expression in vegetative tissues is not always induced upon drought or ABA treatment, which points to an incomplete activation of the transcriptional machinery. It is interesting to note that ectopic expression of the otherwise seed-specific *abi-3* gene product (42) allows the ABA-mediated activation of *Lea* genes in vegetative tissues of *A. thaliana* (100). Similarly, the *CDeT27–45* promoter from *C. plantagineum* was only fully responsive to ABA in *A. thaliana* in the presence of the ABI3 product (39a). These experiments suggest that the ABI3 gene product can functionally interact with different promoters.

Second Messengers and Signaling Molecules

Protein phosphorylation and dephosphorylation (via kinases and phosphorylases, respectively) are major mechanisms of signal integration in eukaryotic cells. Two *A. thaliana* genes encoding calcium-dependent kinases are induced by dehydration (Table 1; 127), which suggests that they may participate in

phosphorylation processes occurring in response to drought. A serine-threonine-type protein kinase has also been isolated from wheat and shows accumulation in ABA-treated embryos and in dehydrated shoots (Table 1; 4). However, the phosphorylation targets of these kinases are not yet known, and their exact roles are obscure.

A role for protein phosphorylation in the drought-stress response is also suggested on the basis of functional studies of the ABA-responsive RAB17 protein from maize (45). This protein is highly phosphorylated in vivo, probably via catalysis by casein kinase 2. The RAB17 protein has been found to be distributed between the cytoplasm and the nucleus of maize embryos, in different states of phosphorylation (5, 45). Biochemical studies showed that RAB17 binds peptides with nuclear localization signals and that the binding is dependent on phosphorylation. It has been suggested that RAB17 mediates the transport of specific nuclear-targeted proteins during stress (45).

Cytoplasmic calcium acts as a second messenger in many cellular processes and may also be involved in the signaling pathways mediating the expression of drought-related genes (13). Stomatal closure is an early plant response to drought, and increases in the cytosolic concentration of free calcium, together with pH changes, are considered to be primary events in the ABA-mediated reduction of stomatal turgor (115). However, it is likely that calcium, together with phosphorylation processes, plays a more general role in the mechanisms associated with drought-stress perception. For example, the *A. thaliana ABI1* gene product is thought to be a calcium-activated phosphoprotein phosphatase (74, 86). Furthermore, a transcript encoding a phosphatidylinositol-specific phospholipase C, an enzyme involved in catalyzing the synthesis of inositol 1,4,5-triphosphate, increases during dehydration (Table 1; 53); inositol-triphosphate stimulates the release of Ca^{2+} from intracellular stores.

Posttranscriptional Control

Much of the effort to understand gene regulation during drought has been devoted to transcriptional mechanisms, but it has become clear that other potential control points include mRNA processing, transcript stability, translation efficiency, and protein modification or turnover. General posttranscriptional mechanisms in plants have recently been reviewed (41, 121). Evidence is emerging that these mechanisms also play a role during stress responses. In *C. plantagineum,* drought stress induces some proteins that are synthesized in a light-dependent manner (see above); for some of these proteins the levels of the mRNA do not parallel those of the proteins, which suggests posttranscriptional regulation (2). A more detailed analysis of alfalfa (*Medicago sativa*) suggests that increased mRNA stability is involved in the accumulation of the MsPRP2 transcript (30). The maize *pMAH9* cDNA clone encodes a transcript that is upregulated by drought. The corresponding protein has RNA-binding

characteristics, which suggests that it may play a role in the selective stabilization of mRNAs (Table 2; 78).

A second major control point appears to be the posttranslational modification of proteins, in which phosphorylation is a key mechanism. For example, phosphorylation is involved in the modification of the fructose-1,6-bisphosphatase in drought-stressed leaves of sugar beet (*Beta vulgaris*) (52). Some of the proteases induced by drought stress (Tables 1 and 2) may also have a function in posttranslational modification. Schaffer & Fischer (113) have hypothesized that a thiol protease, the mRNA of which is cold-induced in tomato, could proteolytically activate certain proteins. This mechanism could also operate during drought stress. It has also been suggested that putative protease inhibitors induced during drought (Table 2) have a role in controlling the activity of endogenous proteases (31).

Downregulation of Genes

Until now, most research has focused on understanding how relevant genes are upregulated during drought stress. However, the response to drought also involves the downregulation of several genes. For example, studies of *C. plantagineum* have revealed that transcripts encoding proteins relevant to photosynthesis are downregulated during the dehydration process and thus possibly reduce photooxidative stress (C Bockel & D Bartels, unpublished data). Jiang et al (59) have also shown that the promoter regions of storage protein genes contain the information for their downregulation during seed desiccation. Furthermore, it has recently been reported that histone H1 transcripts accumulate in response to drought stress in vegetative tissues of tomato, and it was suggested that H1 histones are implicated in the repression of gene expression (E Bray, personal communication).

TRANSGENIC PLANTS ASSESSING GENE FUNCTION

Transgenic plants allow the targeted expression of drought-related genes in vivo and are therefore an excellent system to assess the function and tolerance conferred by the encoded proteins. With ectopic expression of genes involved in controlling ABA biosynthesis, it should also be possible to alter the hormonal balance in vivo and thus to clarify the role of ABA in the drought response. Another purpose for using transgenic plants is to improve drought tolerance in agronomically valuable plants. However, despite extensive research, examples of transgenic plants with improved stress tolerance are scarce (see also 12). A reason for this is that stress tolerance is likely to involve the expression of gene products from several pathways.

The accumulation of low–molecular weight metabolites that act as osmoprotectants is a widespread adaptation to dry, saline, and low-temperature

conditions in many organisms. In engineering plants that synthesize protective osmolytes, microorganisms appear to be useful sources for genes. Transgenic tobacco plants that synthesize and accumulate the sugar alcohol mannitol have been obtained by introducing a bacterial gene that encodes mannitol 1-phosphate dehydrogenase. Plants producing mannitol showed increased salt tolerance (122). Similarly, a freshwater cyanobacterium that was transformed with *E. coli bet* genes produced significant amounts of glycine betaine; this stabilized photosynthetic activity in the presence of sodium chloride, allowing improved growth (94). Tobacco plants that accumulate the polyfructose molecule fructan have been engineered using microbial (*Bacillus subtilis* or *Streptococcus mutans*) fructosyltransferase genes. These plants showed improved growth under polyethylene-mediated drought stress (105), with a positive correlation observed between the level of accumulated fructans and degree of tolerance. The mechanism by which fructans confer tolerance is not known, although a mere osmotic effect seems unlikely.

One consequence of drought and many other stresses is the production of activated oxygen molecules that cause cellular injury, and therefore plants with increased concentrations of oxygen scavengers should show improved performances under nonlethal stress conditions. When tobacco Mn-superoxide dismutase was overexpressed in alfalfa, the plants showed an increased growth rate after freezing stress (85).

Although *Lea*-related genes are upregulated abundantly in most plants during all types of osmotic stress, separate ectopic expression of three different representatives in tobacco did not yield an obvious drought-tolerant phenotype (55). However, this result is perhaps less surprising considering that drought stress does induce an array of different LEA-related proteins in plants. It is also likely that other factors are required for the expression of tolerance where LEA-type proteins are involved.

FUTURE PERSPECTIVES

Despite the many genes that have been identified in association with drought stress, much of the data is descriptive, with the functions of only a few of the encoded proteins established. The production of mutants using an antisense-RNA approach is a powerful technique that should continue to elucidate certain aspects of stress tolerance, but it has been most successful only with well-characterized areas of plant metabolism. It is also difficult to devise screening procedures for useful dehydration-tolerance mutants, because of the array of processes simultaneously affected by drought. Resurrection plants would be an excellent source for mutants with decreased tolerance, but *C. plantagineum,* as well as many other resurrection-plant species, has a polyploid genome and is thus unsuitable. Mutant analyses so far exploited for

drought stress have been with ABA-related mutations, and the power of the approach is shown in the cloning of *Abi1* and *Abi3* (43), which has provided new perspectives. Another valuable approach may be to identify those metabolic steps that are most sensitive to drought stress (a technique used to genetically dissect salt stress in yeast) (116). Such an approach can at least begin to elucidate which gene products are of primary importance.

The plant hormone ABA regulates different aspects of the drought-stress response, and thus the synthesis of pure active ABA analogues (103) may help in the development of probes for ABA-binding proteins, which could then shed some light on primary signals. In contrast with the situation with signal perception, some information is available on *cis*- and *trans*-regulatory factors. Several elements in a promoter need to cooperate with multiple DNA-binding proteins to mediate gene expression. The recently described coupling elements (118) are probably only a beginning in resolving the regulatory network. Little progress has been made with the cloning and analysis of drought-related transcription factors, although a biochemical approach and use of the recently established yeast one- and two-hybrid systems (133) should produce new insights. Regulation at stages beyond transcription must also be further considered, because this could make a major contribution to the final gene expression pattern.

The complexity of drought tolerance apparent throughout this review points to control by multiple genes, and thus the identification of quantitative-trait-loci (QTLs) for drought resistance may well be an effective analytical tool. The approach has just begun to be applied to the environmental-stress responses of plants (126) and is particularly promising considering that saturated DNA–marker maps are now available for both genetic model plants and crop plants.

The molecular analysis of the drought response has arrived at a stage where research can build upon a large collection of characterized genes. The use of novel approaches combining genetic, biochemical, and molecular techniques should provide exciting results in the near future.

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Update on Signaling

Gene Expression and Signal Transduction in Water-Stress Response[1]

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Land plants suffer from dehydration or water stress not only under drought and high-salt-concentration conditions but also under low-temperature conditions. They respond and adapt to water stress to survive these environmental stress conditions. Water stress induces various biochemical and physiological responses in plants. Under water-stress conditions plant cells lose water and decrease turgor pressure. The plant hormone ABA increases as a result of water stress, and ABA has important roles in the tolerance of plants to drought, high salinity, and cold. A number of genes that respond to drought, salt, and cold stress at the transcriptional level have recently been described (for review, see Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). The mRNAs of water-stress-inducible genes decrease when the plants are released from stress conditions, which is consistent with evidence that shows that these genes respond to water stress or dehydration. The functions of some gene products have been predicted from sequence homology with known proteins and are thought to have a role in protecting the cells from water deficit (Ingram and Bartels, 1996; Bray, 1997).

Expression patterns of dehydration-inducible genes are complex. Some genes respond to water stress very rapidly, whereas others are induced slowly after the accumulation of ABA. Most of the genes that respond to drought, salt, and cold stress are also induced by exogenous application of ABA (for review, see Shinozaki and Yamaguchi-Shinozaki, 1996; Bray et al., 1997). It appears that dehydration triggers the production of ABA, which in turn induces various genes. Several genes that are induced by water stress are not responsive to exogenous ABA treatment. These findings suggest the existence of both ABA-independent and ABA-dependent signal transduction cascades between the initial signal of drought or cold stress and the expression of specific genes (Shinozaki and Yamaguchi-Shinozaki, 1996; Bray et al., 1997). Promoter analysis of drought- and cold-inducible genes has identified several *cis*-acting elements that are involved in ABA-dependent and ABA-independent responses to conditions of water stress.

Details of molecular mechanisms regulating responses of plant genes to water stress remain to be discovered, and there are many questions to be examined at the molecular level. These include the sensing mechanisms of water stress or osmotic stress, modulation of the stress signals to cellular signals, transduction of the cellular signals to the nucleus, transcriptional control of stress-inducible genes, and the function and cooperation of stress-inducible genes allowing water-stress tolerance. This *Update* focuses on recent progress toward understanding the signal transduction cascades leading to expression of water-stress-inducible genes. Possible sensors of osmotic stress in plants are discussed based on our knowledge of yeast and bacterial sensors. A glossary of terms is included to facilitate the reading.

GLOSSARY OF TERMS

Promoter Regulatory Elements

ABRE, ABA-responsive element (PyACGTGGC).
G-box, Ubiquitous regulatory elements (CACGTG).
DRE, Dehydration-responsive element (TACCGACAT).
MYBRS, MYB recognition sequence (PyAACPyPu).
MYCRS, MYC recognition sequence (CANNTG).

Proteins That Bind to Promoter Regulatory Elements

bZIP, A family of transcription factors with basic region and Leu-zipper motif.
MYC, A family of transcription factors with basic-helix loop-helix (bHLH) and Leu-zipper motif.
MYB, A family of transcription factors with Trp cluster motif.
VP1, A maize transcriptional activator that is mutated in the viviparous 1 mutant.

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Proteins of the Signal Transduction Pathways

PLC, Phospholipase C that produces two second messengers, inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol.
CDPK, Calcium-dependent protein kinase.
MAPK, Mitogen-activated protein kinase.
MAPKK, A protein kinase that phosphorylates MAPK.
MAPKKK, A protein kinase that phosphorylates MAPKK.
RSK, Ribosomal S6 protein kinase.
Two-component His kinase, Bacterial-type sensory kinase.
14-3-3 protein, A signaling molecule acting by kinase modulation and protein-protein interactions.

FUNCTION OF WATER-STRESS-INDUCIBLE GENES

A variety of genes have been reported to respond to water stress in various species, and the functions for many of the proteins they encode have been predicted from sequence homology with known proteins. Genes induced during water-stress conditions are thought to function not only in protecting cells from water deficit by the production of important metabolic proteins but also in the regulation of genes for signal transduction in the water-stress response (Fig. 1). Thus, these gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance: water channel proteins involved in the movement of water through membranes, the enzymes required for the biosynthesis of various osmoprotectants (sugars, Pro, and Gly-betaine), proteins that may protect macromolecules and membranes (LEA protein, osmotin, antifreeze protein, chaperon, and mRNA binding proteins), proteases for protein turnover (thiol proteases, Clp protease, and ubiquitin), the detoxification enzymes (glutathione *S*-transferase, soluble epoxide hydrolase, catalase, superoxide dismutase, and ascorbate peroxidase). Some of the stress-inducible genes that encode proteins, such as a key enzyme for Pro biosynthesis, were overexpressed in transgenic plants to produce a stress-tolerant phenotype of the plants; this indicates that the gene products really function in stress tolerance (Kavi Kishor et al., 1995). The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response: protein kinases, transcription factors, PLC, and 14-3-3 proteins. Now it becomes more important to elucidate the role of these regulatory proteins for further understanding of plant responses to water deficit. The possible function of the drought-, high-salinity-, and cold-inducible genes were recently reviewed by Ingram and Bartels (1996).

REGULATION OF GENE EXPRESSION BY WATER STRESS

Most water-stress-inducible genes respond to treatment with exogenous ABA, whereas others do not. Analyses of the expression of water-stress-inducible genes by ABA in ABA-deficient (*aba*) or ABA-insensitive (*abi*) Arabidopsis mutants have indicated that some of the stress-inducible

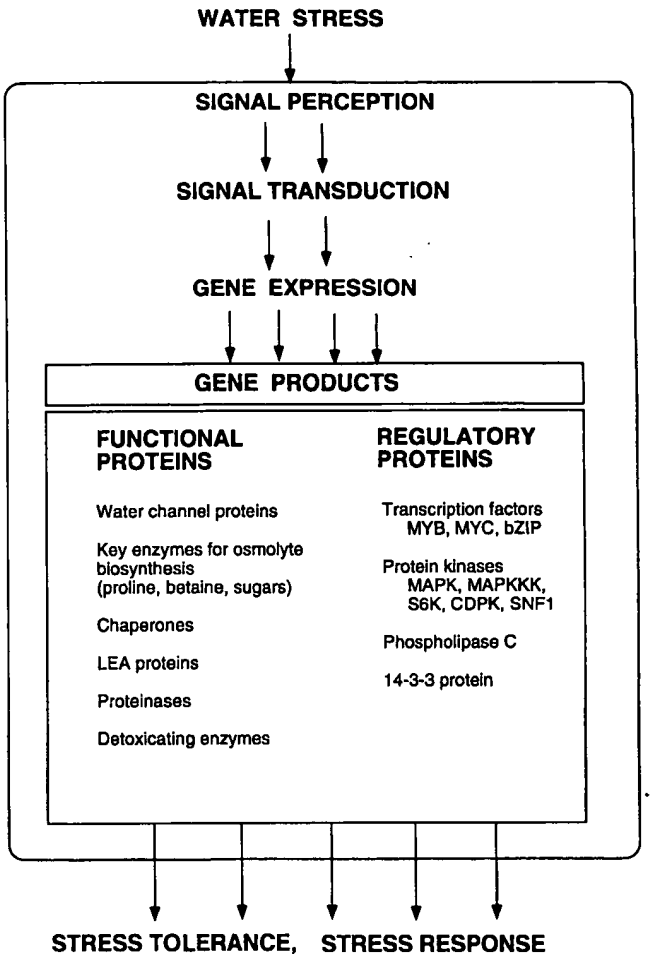

Figure 1. Function of water-stress-inducible gene products in stress tolerance and stress response. The gene products are roughly classified into two groups: functional proteins that are involved in water-stress tolerance and cellular adaptation, and regulatory proteins that may function in gene expression and signal transduction in stress response.

genes do not require an accumulation of endogenous ABA under drought or cold conditions (for review, see Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray et al., 1997). Therefore, there are not only ABA-dependent pathways but also ABA-independent pathways involved in the water-stress response. Analysis of the expression of ABA-inducible genes revealed that several genes require protein biosynthesis for their induction by ABA, suggesting that at least two independent pathways exist between the production of endogenous ABA and gene expression during stress.

As shown in Figure 2, it is now hypothesized that at least four independent signal pathways function in the activation of stress-inducible genes under dehydration conditions (Shinozaki and Yamaguchi-Shinozaki, 1996): two are ABA dependent (pathways I and II) and two are ABA independent (pathways III and IV). One of the ABA-independent pathways overlaps with that of the cold response (pathway IV). One of the ABA-dependent pathways

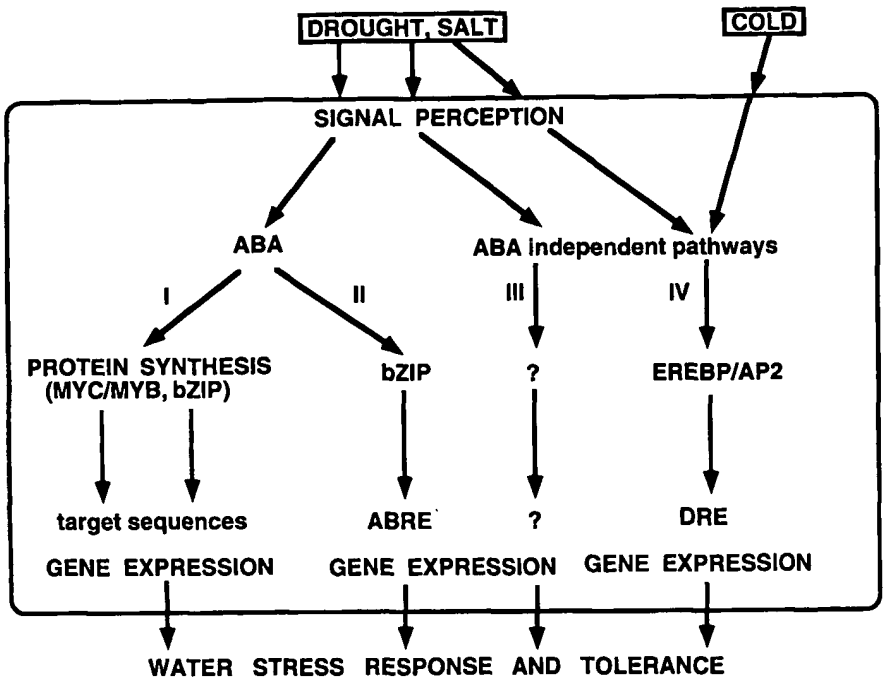

Figure 2. Signal transduction pathways between the perception of a water-stress signal and gene expression. At least four signal transduction pathways exist (I–IV): two are ABA-dependent (I and II) and two are ABA-independent (III and IV). Protein biosynthesis is required in one of the ABA-dependent pathways (I). In another ABA-dependent pathway, ABRE does not require protein biosynthesis (II). In one of the ABA-independent pathways, DRE is involved in the regulation of genes not only by drought and salt but also by cold stress (IV). Another ABA-independent pathway is controlled by drought and salt but not by cold (III).

requires protein biosynthesis (pathway II). Each pathway is discussed separately below.

ABA-Responsive Gene Expression during Water Stress (Pathway II)

Many water-stress-inducible genes are up-regulated by exogenous ABA treatment. The levels of endogenous ABA increase significantly in many plants under drought and high-salinity conditions (Ingram and Bartels, 1996; Bray, 1997). In one of the ABA-dependent pathways (Fig. 2, pathway II), water-stress-inducible genes do not require protein biosynthesis for their expression (for review, see Giraudat et al., 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996). These dehydration-inducible genes contain potential ABREs (PyACGTGGC) in their promoter regions. An ABRE functions as a *cis*-acting DNA element involved in ABA-regulated gene expression. ABREs were first identified in wheat *Em* and rice *rab* genes, and the ABRE-DNA-binding protein EmBP-1 was shown to encode a bZIP protein. The G-box resembles the ABRE motif and functions in the regulation of plant genes in a variety of environmental conditions, such as red light, UV light, anaerobiosis, and wounding. cDNAs for ABRE and G-box-binding proteins have been isolated and have a basic region adjacent to a Leu-zipper motif (bZIP) and constitute a large gene family. Nucleotides around the ACGT core motif have been shown to be involved in determining the binding specificity of bZIP proteins (for review, see Menkens et al., 1995). Furthermore, a coupling element is required to specify the function of the ABRE, constituting an ABA-responsive complex in the regulation of the *HVA22* gene (Shen and Ho, 1995). However, it has not been resolved how ABA activates bZIP proteins to bind to ABRE and initiate transcription of ABA-inducible genes. Further studies are necessary for the precise understanding of the molecular mechanisms of ABA-responsive gene expression that require ABRE as a *cis*-acting element.

There are several *cis*-acting elements other than ABRE that function in ABA-responsive gene expression not only under water-stress conditions but also in seed desiccation. The Sph box and GTGTC motifs regulate ABA- and VP1-dependent expression of the maize *C1* gene, whose product is an MYB-related transcription factor and functions as a controlling element in anthocyanin biosynthesis during seed development (for review, see McCarty, 1995). VP1 encodes a transcriptional activator and is thought to cooperate with bZIP proteins, and the Arabidopsis ABI3 protein has sequence and functional similarity with maize VP1.

ABA-Dependent Gene Expression Requiring Protein Biosynthesis (Pathway I)

In one of the two ABA-dependent pathways (Fig. 2, pathway I), biosynthesis of protein factors is necessary for the expression of water-stress-inducible genes. The induction of an Arabidopsis drought-inducible gene, *rd22*, is mediated by ABA and requires protein biosynthesis for its ABA-dependent expression (Shinozaki and Yamaguchi-Shinozaki, 1996). A 67-bp region of the *rd22* promoter is essential for this ABA-responsive expression and contains several conserved motifs of DNA-binding proteins, such as MYC and MYB, but this region has no ABREs (Iwasaki et al., 1995). A cDNA for a transcription factor MYC homolog, named rd22BP1, was cloned by the DNA-ligand-binding method using the 67-bp DNA as a probe. The *rd22BP1* gene is induced by drought and salt stress. These results suggest that a drought- and salt-inducible MYC homolog might function in the ABA-inducible expression of *rd22* (Abe et al., 1997). The *Atmyb2* gene that encodes a MYB-related

protein is induced by dehydration stress (Urao et al., 1993). High-salt-concentration conditions and application of exogenous ABA also result in the induction of *Atmyb2*, although *Atmyb2* does not respond to cold or heat stress. Recombinant ATMYB2 protein binds to the MYBRS in the 67-bp region of the *rd22* promoter. Therefore, the ATMYB2 protein might also cooperatively function with the rd22BP1 protein as a transcription factor that controls the ABA-dependent expression of the *rd22* gene (Fig. 2, pathway I; Abe et al., 1997).

Several bZIP transcription factors from rice, maize, and Arabidopsis plants (Kusano et al., 1995; Lu et al., 1996; Nakagawa et al., 1996) respond to cold, dehydration, and exogenous ABA treatment. These bZIP proteins bind to G-box-like sequences. These results suggest that ABA-inducible bZIP proteins are also involved in one of the ABA-dependent pathways (Fig. 2, pathway I). Many stress- and ABA-inducible genes encoding various transcription factors have now been reported. These transcription factors are thought to function in the regulation of ABA-inducible genes, which respond to water stress rather slowly after the production of ABA-inducible transcription factors (pathway I).

ABA-Independent Gene Expression during Water Stress (Pathways IV and III)

Several genes are induced by drought, salt, and cold in *aba* (ABA-deficient) or *abi* (ABA-insensitive) Arabidopsis mutants. This suggests that these genes do not require ABA for their expression under cold or drought conditions but do respond to exogenous ABA (for review, see Thomashow, 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). These genes include *rd29A* (*lti78* and *cor78*), *kin1*, *cor6.6* (*kin2*), and *cor47* (*rd17*). Among them, the expression of a drought-inducible gene for *rd29A/lti78/cor78* was extensively analyzed (Yamaguchi-Shinozaki and Shinozaki, 1994). At least two separate regulatory systems function in gene expression during drought and cold stress; one is ABA independent and the other is ABA dependent. A 9-bp conserved sequence, TACCGACAT, termed DRE, is essential for the regulation of the induction of *rd29A* under drought, low-temperature, and high-salt-concentration stress conditions but does not function as an ABRE (Fig. 2, pathway IV). The *rd29A* promoter contains ABRE, which probably functions in ABA-responsive expression. DRE-related motifs have been reported in the promoter regions of many cold- and drought-inducible genes (Thomashow, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). These results suggest that DRE-related motifs, including C-repeat, which contains a CCGAC core motif, are involved in drought- and cold-responsive but ABA-independent gene expression. Protein factor(s) that specifically interact with the 9-bp DRE sequence were detected in a nuclear extract prepared from either dehydrated or untreated Arabidopsis plants (Yamaguchi-Shinozaki and Shinozaki, 1994). Recently, several independent cDNAs for DRE/C-repeat-binding proteins have been cloned (Stockinger et al., 1997; H. Liu, Q. Abe, K. Yamaguchi-Shinozaki, and K. Shinozaki unpublished data) using the yeast one-hybrid-screening method. All of the DRE/C-repeat-binding proteins contain a conserved DNA-binding motif that has also been reported in EREBP and AP2 proteins (EREBP/AP2 motif) that are involved in ethylene-responsive gene expression and floral morphogenesis, respectively. Analyses of the transcriptional control with these DRE/C-repeat-binding proteins will provide a precise mechanism of the ABA-independent pathway in the water-stress response.

There are several drought-inducible genes that do not respond to either cold or ABA treatment, which suggests that there is a fourth pathway in the dehydration-stress response (Fig. 2, pathway III). These genes include *rd19* and *rd21*, which encode different thiol proteases, and *erd1*, which encodes a Clp protease regulatory subunit (Nakashima et al., 1997). Promoter analysis of these genes will give us more information about the pathway III.

SIGNAL TRANSDUCTION IN RESPONSE TO WATER STRESS

Signal transduction cascades from the sensing of water stress signals to the expression of various genes and the signaling molecules that function in the cascade have not been extensively studied in plants and are attractive research subjects. Stomata closure is well characterized as a model system in the responses of plant cells to water stress (Kearns and Assmann, 1993; Giraudat et al., 1994). During stomata closure, the level of cytoplasmic Ca^{2+} is increased, which suggests that Ca^{2+} functions as a second messenger in the osmotic stress response. In animal cells, IP_3 is involved in the release of Ca^{2+} into the cytoplasm from intracellular stores, and it may play a similar role in plant cells. Ca^{2+} and IP_3 are the most probable candidates as second messengers in water-stress responses in plant cells (Fig. 3; for review, see Coté, 1995). Phosphorylation processes are now thought to have important roles in various signal transduction cascades in plants as well as in yeasts and animals. Various protein kinases have been reported in plants and are thought to function in phosphorylation processes in various signal transduction pathways, including water-stress and ABA responses (Fig. 3; Shinozaki and Yamaguchi-Shinozaki, 1996).

Second Messengers

The turgor pressure of plant cells is subject to feedback in response to changes in external osmotic pressure. The cytoplasmic Ca^{2+} signal transduction pathway is involved in turgor regulation in plant cells (for review, see Coté, 1995; Niu et al., 1995). An increase of cytoplasmic Ca^{2+} serves to stimulate ion transport pathways under hypo-osmotic stress. Stomata closure is induced by the release of Ca^{2+} into the cytoplasm. Phosphoinositide signaling has been implicated in the elevation of cytoplasmic Ca^{2+} in guard cells because artificial elevation of IP_3 in the cytoplasm results in Ca^{2+} mobilization (Blatt et al., 1990; Gilroy et al., 1990). The IP_3 content has been demonstrated to increase following hyperosmotic stress. There are changes in the levels of precursor lipids to IP_3 and in the activity of

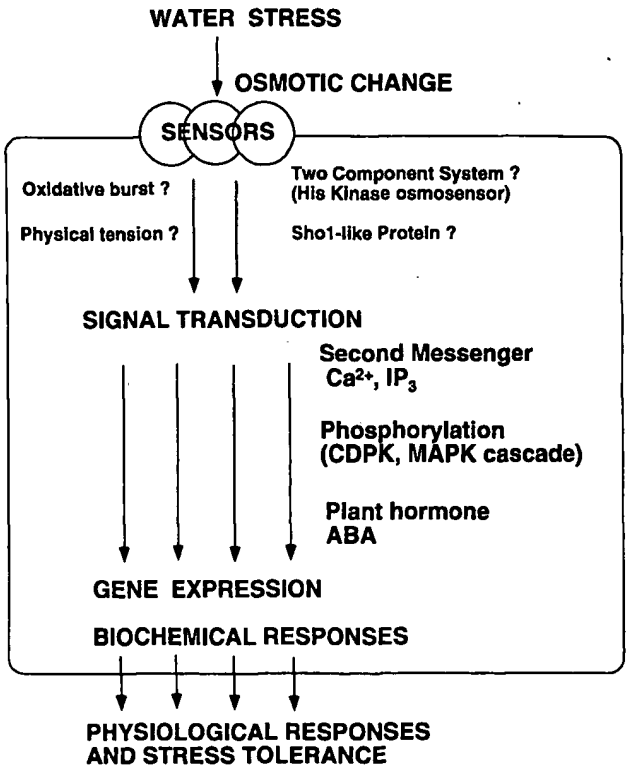

Figure 3. Second messengers and factors involved in the signal perception and the signal transduction in water-stress response. Two-component His kinase is thought to function as an osmosensor in plants. Ca^{2+} and IP_3 are the most probable second messengers of the dehydration signal. The phosphorylation process functions in water-stress and ABA signal transduction pathways. ABA plays important roles in the regulation of gene expression as well as physiological responses during water stress.

enzymes involved in the metabolism of inositol phospholipids after hyperosmotic stress. Phosphatidylinositol 4,5-bisphosphate levels decrease and IP_3 levels increase prior to ABA-induced stomatal closure in guard cells. The competence of vacuoles to respond to IP_3 is enhanced by hyperosmotic stress. These observations provide more evidence for the role of phosphoinositide signaling in osmotic responses.

Cytoplasmic pH is another possible second messenger of ABA signaling in guard cells, and it functions in the Ca^{2+}-independent pathway. ABA evokes an alkalization of the cytoplasm of guard cells, and this has a relationship with the activation of outward-rectifying K^+ channels by ABA (for review, see Giraudat et al., 1994).

Water-Stress-Inducible Genes for Signaling Factors

In higher plants many genes involved in signal transduction pathways, such as those encoding for calmodulins, G-proteins, protein kinases, and transcription factors, are induced by environmental stimuli. The genes for several protein kinases and for PLC are also induced by drought, salt, and cold stress (Shinozaki and Yamaguchi-Shinozaki, 1996).

A cDNA for PLC, AtPLC1, was isolated from dehydrated Arabidopsis (Hirayama et al., 1995). The *AtPLC1* gene is strongly induced by salt and drought and slightly induced by cold at the transcriptional level. Moreover, two genes for the CDPKs, *ATCDPK1* and *ATCDPK2*, have been demonstrated to be rapidly induced by drought and salt stresses in Arabidopsis (Urao et al., 1994). The stress-inducible PLC and CDPKs might function in the signal transduction cascade under water stress (Fig. 3). In animals, PLC digests phosphatidylinositol 4,5-bisphosphate to generate two second messengers, IP_3 and 1,2-diacylglycerol. IP_3 induces the release of Ca^{2+} into the cytoplasm, which in turn causes various responses in the cytoplasm. In plants a similar system may function in the water-stress response. Recently, co-expression of the constitutively active catalytic domain of a stress-inducible CDPK, ATCDPK1, was demonstrated to induce the expression of an ABA-inducible HVA1 promoter-reporter fusion gene in maize protoplasts (Sheen, 1996). The HVA1 promoter is also activated not only by cold, high salt, and ABA treatment but also by Ca^{2+} in protoplasts. These observations also supportthat Ca^{2+} might function as a second messenger and that ATCDPK1 functions as a positive regulator in the signal transduction pathways under water-stress conditions in plants.

MAPK is involved in the signal transduction pathways associated not only with growth-factor-dependent cell proliferation but also with environmental stress responses in yeast and animals. Many genes for protein kinases involved in MAPK cascades have been identified. There are at least four subfamilies of MAPK based on phylogenetic analysis (for review, see Mizoguchi et al., 1997). One of the MAPK genes, ATMPK3, is induced at the mRNA level by drought, low temperature, high salinity, and touch (Mizoguchi et al., 1996). Moreover, two genes for protein kinases involved in the MAPK cascade, MAPKKK (ATMEKK1) and ribosomal S6 kinase (RSK; ATPK19), are induced by similar stresses. Recently, alfalfa MAPK, MMK4, was demonstrated to be activated at posttranslational levels by a variety of stresses, including drought, low temperature, and mechanical stimuli (Jonak et al., 1996). The MMK4 gene is also induced by these stresses at the transcriptional level. These observations indicate that the MAPK cascades might function in the signal transduction pathways in the water-stress response (Fig. 3). In *Saccharomyces cerevisiae*, one of the MAPK cascades (Ssk2/Ssk22, Pbs2, and Hog1) functions in response and adaptation to high osmolarity (for review, see Wurgler-Murphy and Saito, 1997). Furthermore, the mammalian MAPKs p38 and JNK1 can functionally complement yeast *hog1*. These MAPKs are parts of the MAPK cascades that are activated by various stresses, including high osmolarity. There are several other signaling molecules of which genes are up-regulated by water stress (Fig. 1; Shinozaki and Yamaguchi-Shinozaki, 1996). Their roles in the water-stress response have not yet been elucidated.

ABA Signal Transduction

The role of ABA in water-stress signal transduction has been analyzed with ABA-insensitive mutants in various species. Of these, maize VP1 and Arabidopsis *abi1*, *abi2*, and *abi3* have been extensively characterized and their genes cloned. Among them, ABI1 and ABI2 gene products function mainly in vegetative tissues and also participate to some extent in seed development. Because of the wilty phenotypes of *abi1* and *abi2* mutants, ABI1 and ABI2 are thought to have important roles in ABA-dependent signal transduction pathways during water stress (for review, see Giraudat et al., 1994). The *ABI1* and *ABI2* genes have been cloned and shown to encode proteins that are related to type 2C protein Ser/Thr phosphatases (PP2Cs) (Leung et al., 1994, 1997; Meyer et al., 1994). The *ABI1* gene product functions in stomata closure, and the *abi1* plant accordingly has a wilty phenotype (Armstrong et al., 1995). ABI1 was demonstrated to function as a negative regulator in ABA-dependent gene expression in a transient expression experiment in which maize protoplasts were used (Sheen, 1996). By contrast, the dehydration-inducible ATCDPK1 encoding CDPK functions as a positive regulator in this regulation. These results indicate that a protein phosphorylation and dephosphorylation process might be involved in ABA-responsive signaling during water deficit. Recently, ABA was shown to induce a rapid and transient activation of MAPK in barley aleurone protoplasts (Knetsch et al., 1996). Correlation between ABA-induced MAPK activation and ABA-induced gene expression implies that MAPK might be involved in ABA signal transduction (Fig. 3). Another Arabidopsis mutant, *era*, that confers an enhanced response to exogenous ABA, has mutations in the *ERA1* gene, which encodes the β-subunit of farnesyl transferase (Culter et al., 1996). This suggests that a negative regulator of ABA sensitivity may require farnesylation to function.

Under water-stress conditions ABA is synthesized de novo, and this increase in ABA level requires protein biosynthesis. As mentioned above, this process is important for drought-inducible gene expression. Many ABA-deficient mutants that do not produce ABA have been isolated in various plants. Recently, an ABA-deficient tobacco mutant, *aba2*, was isolated by transposon-tagging using the maize Ac transposon (Marin et al., 1996). The ABA2 cDNA encodes a chloroplast-imported protein that exhibits zeaxanthin epoxidase activity, which functions in the first step of the ABA biosynthesis pathway. The tobacco ABA2 gene corresponds to the Arabidopsis ABA1 gene. Recently, Arabidopsis *aba2* and *aba3* mutants were isolated. Molecular analysis of the expression of these genes will aid the study of the regulation of ABA biosynthesis and ABA-dependent gene expression during water stress (Fig. 2).

SIGNAL PERCEPTION AND SENSORS OF WATER STRESS

Water deficits occur not only during drought and under conditions of high salt concentrations but also during cold conditions. They probably also cause the decrease of turgor pressure at the cellular level. A change in the osmotic potential across a plasma membrane, caused by the decrease of turgor pressure, might be a major trigger of the water-stress response at the molecular level. Osmosensors of yeasts have been extensively studied (for review, see Wurgler-Murphy and Saito, 1977), and cloning of osmosensors involved in the signal perception of water stress in plants is in progress based on the knowledge of osmosensors in yeast.

Osmosensors

The "two-component system" is known to be widespread and involved in various signal transduction pathways in bacteria. In *Escherichia coli*, a two-component system is involved in sensing osmotic change and osmotic responses. EnvZ, a two-component His kinase, functions as an "osmosensor," or a "sensory kinase," and monitors mechanical changes of the plasma membrane during osmotic stress (for review, see Wurgler-Murphy and Saito, 1997). EnvZ is activated by autophosphorylation at a His residue under hyperosmotic conditions and then phosphorylates an Asp residue of the OmpR protein, a "response regulator." Phosphorylated OmpR functions as a transcription factor to up-regulate the OmpC gene and down-regulate the OmpF gene. Both genes encode proteins of the bacterial outer membrane, and together these proteins regulate turgor pressure.

In yeast, exposure to high osmolarity activates a MAPK cascade that includes PBS2 (MAPKK) and HOG1 (MAPK) and then activates several genes involved in the biosynthesis of glycerol, which is an important osmoprotectant. Three gene products (Sln1p, Ypd1p, and Ssk1p) that act in an early phase of the hyperosmolarity-stress response encode signaling molecules that constitute a prokaryote-type two-component regulatory system (Posas et al., 1996; for review, see Wurgler-Murphy and Saito, 1997). Sln1p is thought to act as a sensor protein, phosphorylating response regulator proteins Ypd1 and Ssk1p under conditions of high osmolarity. The three protein factors perform a four-step phosphorelay (His-Asp-His-Asp). At high osmolarity phosphorylated Ssk1p activates Ssk2p or Ssk22p (MAPKKKs; Maeda et al., 1995), which results in the activation of Pbs2p (MAPKK) by Ser-Thr phosphorylation. Then, phosphorylated Pbs2p activates Hog1p (MAPK) by Thr-Tyr phosphorylation. A similar osmosensing mechanism might operate in higher plants in response to a water deficit. One of the two-component His kinases might also function as a osmosensor in water-stress response in higher plants because an Arabidopsis SLN1 homolog, ATHK1, was recently shown to complement yeast sln1 mutants and functions as a osmosensor in yeast (T. Urao, K. Yamaguchi-Shinozaki, and K. Shinozaki, unpublished data; Fig. 3). In higher plants another two-component His kinase, ETR1, is a receptor in ethylene signal transduction (Chang, 1996). Two-component His kinases may function as sensors or receptors in various signal transduction pathways in plants.

Another transmembrane osmosensor, Sho1p, has been reported by Maeda et al. (1995). Sho1p contains four closely packed hydrophobic transmembrane peptides. The COOH-

terminal region contains an SH3 domain that modulates various signal transduction pathways. Under conditions of high osmolarity, Sho1p activates the PBS2-HOG1 MAPK cascade. A Sho1p-like membrane protein might be another candidate as an osmosensor in plants (Fig. 3).

Other Cellular Triggers of Water-Stress Responses

Drought stress induces genes for detoxification enzymes, such as ascorbate peroxidase, superoxide dismutase, glutathione *S*-transferases, and soluble epoxide hydrolase (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996). Cold stress also induces similar genes. An oxidative burst might function as one of the triggers of the water-stress responses.

In addition, change in the physical tension of cytoskeletons during water stress might be one of the triggers of osmotic responses. Some of the water-stress-inducible genes are also induced by touch (Mizoguchi et al., 1997). Touch not only induces the release of Ca^{2+} in the cytoplasm but also induces many genes, named touch genes, such as calmodulins, Ca^{2+}-binding proteins, xyloglucan endotransglycosylase, and protein kinases involved in the MAPK cascade. However, the sensing mechanism of oxidative burst or touch have not yet been identified.

CONCLUSIONS AND FUTURE PERSPECTIVES

Many genes that are regulated by water stress have been reported in a variety of plants. Analyses of stress-inducible gene expression have revealed the presence of multiple signal transduction pathways between the perception of water stress and gene expression. This explains the complex stress response observed after exposure of plants to drought, salt, and cold. At least four different transcription factors have been suggested to function in the regulation of dehydration-inducible genes; two are ABA responsive and two are ABA independent. The transcriptional regulatory regions of the dehydration-induced genes have been analyzed to identify several *cis*- and *trans*-acting elements that are involved in the water-stress response. A newly identified DRE *cis* element functions in the regulation of rapidly inducible genes in an ABA-independent manner. ABRE functions in the induction of genes after the accumulation of ABA during water stress. Several genes for transcription factors are induced by water stress and ABA at transcriptional levels, which might be involved in the regulation of slowly induced stress-involved genes. In addition, many genes for factors involved in the signal transduction cascades, such as protein kinases and PLC, are regulated by water-stress signals (Shinozaki and Yamaguchi-Shinozaki 1996; Mizoguchi et al., 1997). These signaling factors might be involved in the amplification of the stress signals and adaptation of plant cells to water-stress conditions. Based on the knowledge of osmosensors in yeasts and bacteria, cloning of homologs of the two-component His kinase as osmosensors in higher plants is in progress.

Molecular analyses of these factors should provide a better understanding of the signal transduction cascades during water stress. Transgenic plants that modify the expression of these genes will give more information about the function of their gene products. Recently, mutants that had a resistant or a sensitive phenotype to water stress were reported. Isolation of these mutant genes will give more information concerning factors involved in the signal transduction cascades and sensors. A combination of genetic and molecular approaches will give more insight into the molecular mechanisms of water-stress responses in plants.

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Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought- and Low-Temperature-Responsive Gene Expression, Respectively, in Arabidopsis

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Plant growth is greatly affected by drought and low temperature. Expression of a number of genes is induced by both drought and low temperature, although these stresses are quite different. Previous experiments have established that a *cis*-acting element named DRE (for dehydration-responsive element) plays an important role in both dehydration- and low-temperature-induced gene expression in Arabidopsis. Two cDNA clones that encode DRE binding proteins, DREB1A and DREB2A, were isolated by using the yeast one-hybrid screening technique. The two cDNA libraries were prepared from dehydrated and cold-treated rosette plants, respectively. The deduced amino acid sequences of DREB1A and DREB2A showed no significant sequence similarity, except in the conserved DNA binding domains found in the EREBP and APETALA2 proteins that function in ethylene-responsive expression and floral morphogenesis, respectively. Both the DREB1A and DREB2A proteins specifically bound to the DRE sequence in vitro and activated the transcription of the β-glucuronidase reporter gene driven by the DRE sequence in Arabidopsis leaf protoplasts. Expression of the *DREB1A* gene and its two homologs was induced by low-temperature stress, whereas expression of the *DREB2A* gene and its single homolog was induced by dehydration. Overexpression of the DREB1A cDNA in transgenic Arabidopsis plants not only induced strong expression of the target genes under unstressed conditions but also caused dwarfed phenotypes in the transgenic plants. These transgenic plants also revealed freezing and dehydration tolerance. In contrast, overexpression of the DREB2A cDNA induced weak expression of the target genes under unstressed conditions and caused growth retardation of the transgenic plants. These results indicate that two independent families of DREB proteins, DREB1 and DREB2, function as *trans*-acting factors in two separate signal transduction pathways under low-temperature and dehydration conditions, respectively.

INTRODUCTION

Drought and low temperature are adverse environmental conditions that affect the growth of plants and the productivity of crops. However, it has been suggested that plants have common mechanisms in their physiological responses and tolerance to drought and low temperature. For example, abscisic acid (ABA) is produced under both drought and low-temperature stresses and plays important roles in allowing plants to tolerate both stresses. Also, plants grown under dehydration conditions show higher tolerance to low-temperature stress than do well-watered plants.

A number of genes have been described that respond to both drought and low-temperature stress at the transcriptional level (reviewed in Thomashow, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). The functions of some gene products have been predicted from sequence homology with known proteins and are thought to play a role in protecting the cells from water deficit and low temperature (reviewed in Thomashow, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). Most of the drought- and cold stress–inducible genes that have been studied to date are also induced by ABA. Dehydration appears to trigger the production of ABA, which in turn induces expression of various

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genes. *cis*- and *trans*-acting factors involved in ABA-induced gene expression have been analyzed extensively (reviewed in Chandler and Robertson, 1994; Giraudat et al., 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997).

However, expression of several ABA-inducible genes is induced by both cold and drought in ABA-deficient (*aba*) and ABA-insensitive (*abi*) Arabidopsis mutants. This suggests that these genes do not require ABA for their expression under cold and drought conditions but do respond to ABA (reviewed in Thomashow, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). These genes include *rd29A*/*lti78*/*cor78*, *kin1*, *cor6.6*/*kin2*, and *cor47*/*rd17* (Nordin et al., 1991; Kurkela and Borg-Franck, 1992; Horvath et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993; Iwasaki et al., 1997). The promoter region of the *rd29A* gene was analyzed, and a novel *cis*-acting element responsible for dehydration- and cold-induced expression was identified at the nucleotide sequence level by using transgenic plants (Yamaguchi-Shinozaki and Shinozaki, 1994). A 9-bp conserved sequence, TACCGACAT, termed the dehydration-responsive element (DRE), is essential for the regulation of dehydration-responsive gene expression. The DRE has been demonstrated to function as a *cis*-acting element involved in the induction of *rd29A* expression by low-temperature stress.

DRE-related motifs have been reported in the promoter regions of cold- and drought-inducible genes such as *kin1*, *cor6.6*, and *rd17* (Wang et al., 1995; Iwasaki et al., 1997). A similar motif was also reported (C repeat; TGGCCGAC) in the promoter region of cold-inducible *cor15a* (Baker et al., 1994). The CCGAC core sequence was found in the promoter regions of the cold-inducible oilseed rape gene *BN115* and designated the low-temperature-responsive element (Jiang et al., 1996). These results suggest that DRE-related motifs are involved in both drought- and cold-responsive but ABA-independent gene expression.

It is important to understand how two different stress signals, drought and cold, are transmitted separately in plant cells to activate DRE-dependent transcription of the *rd29A*/*cor78* gene. For this purpose, it is critical to identify *trans*-acting factors that regulate DRE-dependent gene expression. We attempted to isolate cDNAs for DRE binding proteins by using the DNA ligand binding screening method, but we were not successful. Next, we tried to isolate cDNAs for DRE binding proteins by using the yeast one-hybrid screening system. Meanwhile, Stockinger et al. (1997) reported cloning a cDNA (named CBF1) for a C repeat/DRE binding protein from Arabidopsis by using yeast one-hybrid screening. The CBF1 protein has a DNA binding motif found in tobacco EREBP1 (Ohme-Takagi and Shinshi, 1995), which is involved in ethylene-responsive gene expression, and in Arabidopsis APETALA2 (AP2; Jofuku et al., 1994), which is involved in floral morphogenesis. The CBF1 protein can bind to the C repeat/DRE motif in the *cor15a* promoter and function as a *trans*-activator in yeast. However, the CBF1 cDNA clone was isolated from a cDNA library prepared from unstressed normally grown Arabidopsis plants, and it had an abnormally fused structure with the 25S rRNA gene (Stockinger et al., 1997).

In contrast, we cloned two different cDNAs encoding DRE binding proteins (DREB1A and DREB2A) of Arabidopsis that specifically interact with the DRE sequence in the promoter region of the *rd29A* gene from dehydrated and low-temperature-treated Arabidopsis plants by using the yeast one-hybrid screening method. Genes encoding the DREB1A protein and its two homologs were induced to express by cold stress; genes encoding the DREB2A protein and its single homolog rapidly were induced to express by dehydration and high-salt stress. Both the DREB1A and DREB2A homologs contain the EREBP/AP2 DNA binding domain like that of CBF1. We analyzed the function of the DREB1A and DREB2A proteins as *trans*-acting factors by using transient expression in Arabidopsis leaf protoplasts and overexpression in transgenic Arabidopsis plants. We discuss the different functions of the DREB1A and DREB2A proteins in the separation of two signaling pathways under cold and dehydration stress conditions in ABA-independent gene expression in vegetative tissues.

RESULTS

Isolation of cDNAs Encoding DNA Binding Proteins That Recognize DRE in the 71-bp DNA Fragment of the *rd29A* Promoter

To isolate cDNAs encoding DNA binding proteins that interact with the DRE motif, we used the yeast one-hybrid screening system. We first constructed a parental yeast strain carrying as dual reporter genes integrated copies of *HIS3* and *lacZ* with four-times tandemly repeated 71-bp DNA fragments of the *rd29A* promoter upstream of the TATA element (Figure 1). The 71-bp fragment contains a DRE motif at center. The resulting yeast strain transcribes the *HIS3* gene at basal levels, grows on media lacking histidine (but not in the presence of 10 mM 3-aminotriazole [3-AT], a competitive inhibitor of the *HIS3* gene product), and forms white colonies on filter papers containing X-gal. The yeast cells were then separately transformed with three expression libraries of cDNA fragments of mRNAs prepared from Arabidopsis rosette plants that had been dehydrated for 2 hr, cold treated for 24 hr, or undehydrated. The cDNA fragments were fused to the transcriptional activation domain of the yeast GAL4 (Figure 1).

We screened 1.8×10^6, 1.2×10^6, and 1.5×10^6 yeast transformants of libraries prepared from 2-hr dehydrated, 24-hr cold-treated, and unstressed Arabidopsis rosette plants, respectively. Clones 2 to 41 and clone 1 were 3-AT resistant and isolated from libraries prepared from 2-hr dehydrated and 24-hr cold-treated plants, respectively (Table 1). All of the isolated cDNA clones induced *lacZ* activity and formed blue colonies on filter papers containing X-gal. The

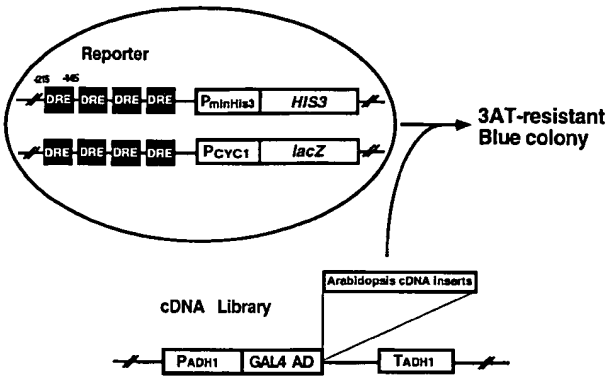

Figure 1. Strategy for the Isolation of cDNAs Encoding DRE Binding Proteins by Selection in Yeast.

An expression library of hybrid proteins was transformed into the yeast strain carrying dual reporter genes *HIS3* and *lacZ* under the control of the 71-bp promoter region of *rd29A* containing the DRE. The hybrids contain protein coding sequences fused to the end of the GAL4 activation domain (AD). Hybrid proteins that recognize the binding site act as transcriptional activators of the reporter genes, allow the cells to grow in the presence of 3-AT (a competitive inhibitor of the *HIS3* gene product), and turn the cells blue in β-galactosidase assay. $P_{minHis3}$ indicates the minimal promoter of the *HIS3* gene, and P_{CYC1} indicates the minimal promoter of the yeast cyclin gene. PADH1 indicates the promoter of the alcohol dehydrogenase1 (*ADH1*) gene, and TADH1 indicates the terminator of the *ADH1* gene.

cDNA fragments of the isolated plasmids were analyzed by restriction enzyme digestion and DNA sequencing, which led to the classification of these 41 cDNA clones into seven distinct cDNA groups. Among the seven groups, clone 18 was most abundant in the cDNA library prepared from dehydrated plants (Table 1).

To select cDNAs that encode transcriptional activators in the seven independent cDNA clones that were isolated, the insert cDNA fragments were cloned into the yeast expression vector YepGAP (Figure 2A). Plasmids containing each insert DNA fragment were transformed into yeast strains carrying the dual reporter genes *HIS3* and *lacZ* that had been fused to 71-bp DNA fragments of the *rd29A* promoter containing the DRE sequence. Yeast cells carrying the plasmid containing the cDNA inserts of clones 1 and 18 grew on medium lacking histidine in the presence of 10 mM 3-AT, but yeast cells carrying the plasmid containing the cDNA inserts of five other clones did not. Both the 3-AT–resistant yeast strains also induced *lacZ* activity and formed blue colonies (Figure 2B). In contrast, when plasmids containing the DNA insert of clone 1 or 18 were transformed into yeast strains carrying the dual reporter genes fused to the 71-bp DNA fragment with base substitutions in the DRE sequence mDRE, the yeast strains neither grew on media lacking histidine in the presence of 10 mM 3-AT nor induced *lacZ* activity (Figure 2C). These data indicate that cDNA clones 1 and 18 encode polypeptides that specifically bind to the DRE sequence and activate the transcription of the dual reporter genes in yeast. cDNA clones 1 and 18 were designated DREB1A and DREB2A, respectively, and analyzed further.

Structural Analysis of the DREB1A and DREB2A cDNAs

To examine the structures of the DREB1A and DREB2A cDNA clones, we sequenced inserted DNA fragments of 0.9 and 1.4 kb, respectively. The DREB1A cDNA contains a single open reading frame of 216 amino acids and encodes a putative protein with a predicted molecular mass of 24.2 kD (Figure 3). The DREB2A cDNA contains an open reading frame of 335 amino acids and encodes a putative protein with a predicted molecular mass of 37.7 kD (Figure 3).

We searched DNA and protein databases for sequences homologous to those of the DREB1A and DREB2A proteins and found that each DREB protein has a conserved DNA binding domain of 58 amino acids present in a large family of plant genes for DNA binding proteins, including EREBPs of tobacco and AP2 of Arabidopsis (Figure 4). The deduced amino acid sequences of DREB1A and DREB2A showed no significant sequence identity except in the conserved DNA binding domain. However, each DREB protein contains a basic region in its N-terminal region that might function as a nuclear localization signal and an acidic C-terminal region that might act as an activation domain for transcription. These data suggest that each DREB cDNA encodes a DNA binding protein that might function as a transcriptional activator in plants.

DNA Binding Regions of the DREB1A and DREB2A Proteins Bind Specifically to the DRE Sequence of the *rd29A* Promoter

To identify the target sequence of the DREB1A and DREB2A proteins, the 143 and 166 amino acids of the DNA binding

Table 1. General Characteristics of the cDNA Clones Isolated in This Study[a]

Group	Clone	Insert Size (kb)	No. of Clones Obtained
1	1	0.94	1
2	18	1.4	35
3	63	1.8	1
4	75	0.48	1
5	104	0.77	1
6	125	0.48	1
7	127	1.2	1

[a] Forty-one positive clones were isolated from libraries prepared from dehydrated and cold-treated plants. These 41 cDNA clones were divided into seven distinct cDNA groups.

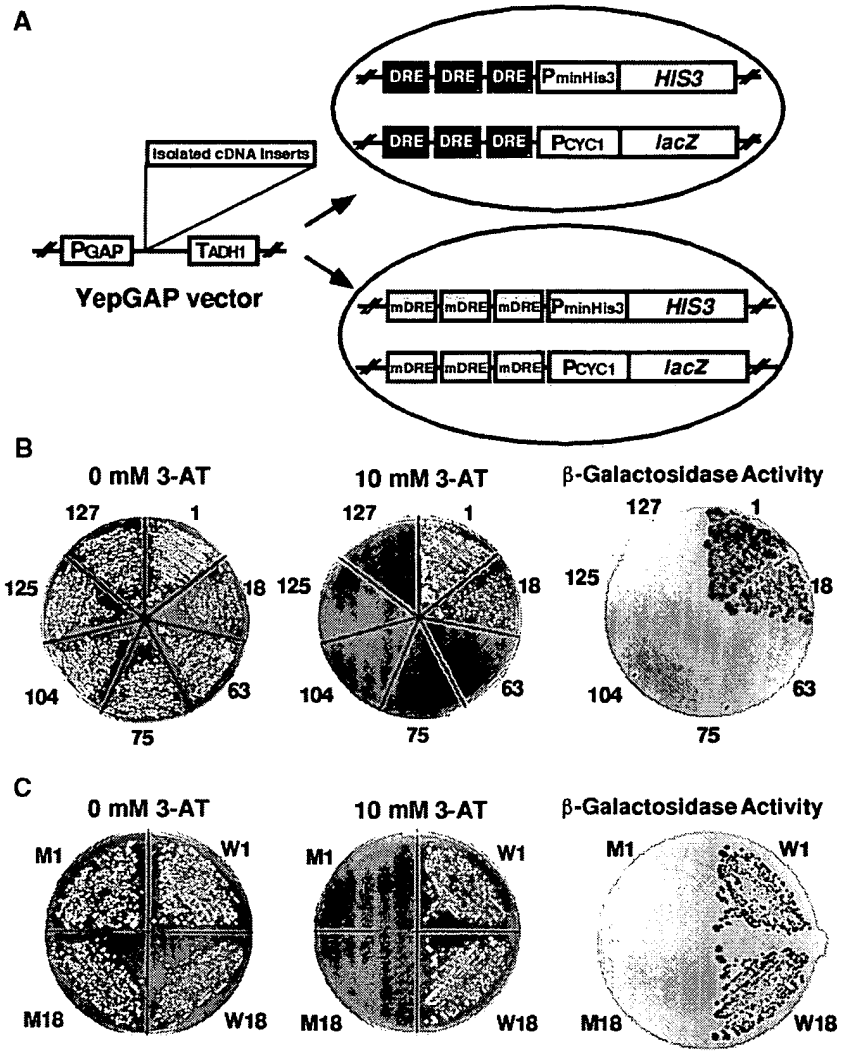

Figure 2. Activation of Dual Reporter Genes in Yeast by Proteins Encoded by Isolated cDNAs.

(A) The insert DNA fragments of the isolated cDNA clones were cloned into the yeast expression vector YepGAP and used for transformation into yeast carrying the dual reporter genes *HIS3* and *lacZ* under the control of the 71-bp promoter region containing the DRE or the 71-bp promoter region containing a mutated DRE (mDRE; M2 in Figure 5). Other abbreviations are as given in the legend to Figure 1. P_{GAP} indicates the promoter of the glyceraldehyde 3-phosphate dehydrogenase gene. TADH1 indicates the terminator of the *ADH1* gene.

(B) All of the yeast transformants carrying dual reporter genes under the control of the wild-type 71-bp promoter region were examined for growth in the presence of 3-AT and β-galactosidase activity. Numbers indicate isolated clone names.

(C) Two plasmids containing insert DNA from clones 1 and 18 were transformed into yeast strains carrying the dual reporter genes under the control of the 71-bp promoter region containing the DRE (W1 and W18) or the mutated DRE sequence (M1 and M18). The transformants were examined for growth in the presence of 3-AT and β-galactosidase activity.

domains of DREB1A and DREB2A, respectively, were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*. The ability of the DREB1A and DREB2A fusion proteins to bind the wild-type or mutated DRE sequences was examined using the gel retardation method. As shown in Figure 5B, both the recombinant DREB1A and DREB2A fusion proteins bound the wild-type 71-bp DNA fragment but not the base-substituted 71-bp fragments M1, M2, and M3. By contrast, both of the fusion proteins bound to the base-substituted 71-bp fragments M4 and M5. The DRE sequence was base-substituted in M1, M2, and M3 but not in M4 and M5 (Figure 5A). These results indicate that the binding of the DREB1A and DREB2A fusion proteins to the DRE sequence is highly specific.

Expression of the *DREB1A* and *DREB2A* Genes

The expression patterns of the *DREB1A* and *DREB2A* genes were analyzed using RNA gel blot hybridization to compare them with that of the *rd29A* gene (Figure 6A). *DREB2A* gene expression was induced within 10 min after dehydration began, and *DREB2A* was strongly expressed after 2 hr. However, there was no significant *DREB1A* mRNA accumulation within 24 hr. There was significant *DREB2A* mRNA accumulation within 10 min after high-salt treatment, whereas the *DREB1A* mRNA was not accumulated (Figure 6A). When, as a control, the plants were transferred from agar to water, rapid but low-level accumulation of *DREB2A* mRNA was detected, whereas *DREB1A* mRNA accumulation was not apparent. Similar results were obtained for the exogenous ABA treatment. By contrast, the *DREB1A* gene was induced to express within 1 hr after exposure to low temperature (4°C), and the level of the *DREB1A* mRNA peaked after 2 hr. However, *DREB2A* mRNA did not accumulate significantly within 24 hr after exposure to low temperature (Figure 6A). These results indicate that the transcription of the *DREB1A* gene is activated by cold stress and that that of the *DREB2A* gene is activated by dehydration and high-salt stress.

Expression of the *rd29A* gene was induced within 20 min by dehydration, and the gene was strongly expressed after 2 hr (Figure 6A). The *rd29A* gene also was induced to express by low-temperature treatment within 2 hr and was strongly expressed after 5 hr. Rapid and strong expression of the *rd29A* gene was observed within 10 min after the initiation of ABA treatment as well as by high-salt treatment. When plants were transferred from agar to water, rapid but weak expression of the *rd29A* gene was detected. The expression of the *DREB1A* gene during cold stress preceded that of the *rd29A* gene (Figure 6A). In contrast, the expression pattern of the *DREB2A* gene during dehydration and high-salt stress was similar to that of the *rd29A* gene. We could detect both *DREB2A* mRNA and *rd29A* mRNA but not *DREB1A* mRNA in all of the tissues of unstressed plants (Figure 6B).

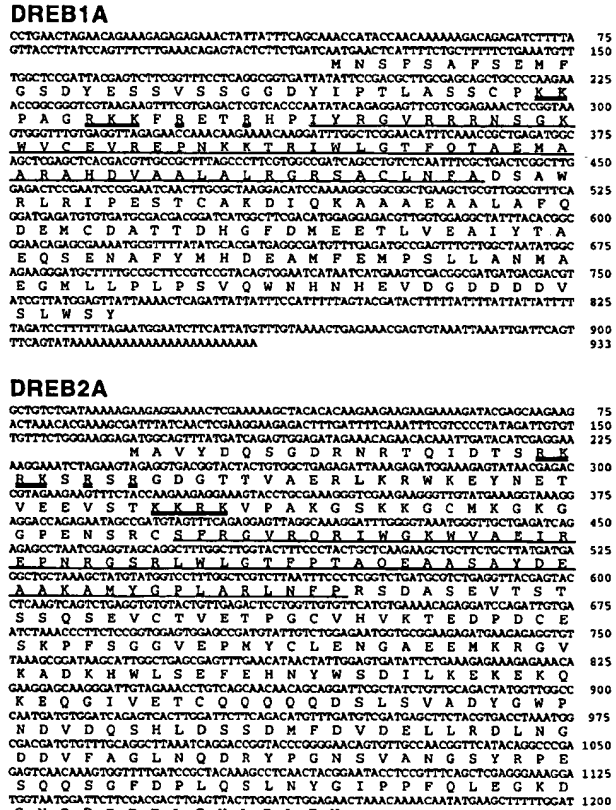

Figure 3. Full-Length Sequences of DREB1A and DREB2A cDNAs.

The EREBP/AP2 domains are underlined. Each DREB protein contains a basic region in the N-terminal region that might function as a nuclear localization signal (double underlines) and an acidic C-terminal region that might act as a transcriptional activation domain. The DREB1A and DREB2A cDNA sequences have been submitted to the GenBank, EMBL, and DDBJ data bases with accession numbers AB007787 and AB007790, respectively.

Isolation of cDNAs That Encode Homologs of the DREB1A and DREB2A Proteins

The number of DREB-related genes in the Arabidopsis genome was estimated by DNA gel blot analysis (Figure 7). Nuclear DNA from Arabidopsis was digested with BamHI, EcoRI, and HindIII and hybridized under both high- and low-stringency conditions by using the DREB1A and DREB2A cDNA inserts as probes. Under high-stringency hybridization conditions, each probe hybridized with a few bands of DNA fragments. Under low-stringency conditions, a few additional bands were detected, suggesting that there may be a few DREB-related genes in the Arabidopsis genome.

To isolate cDNAs for the DREB-related genes, three λgt11 cDNA libraries prepared from dehydrated, cold-treated, and unstressed plants, respectively, were screened with the DREB1A and DREB2A cDNA inserts as probes. Three independent cDNA clones were isolated using the DREB1A cDNA as a probe; one is identical to DREB1A and the other two are homologs (named DREB1B and DREB1C). The DREB1B clone is identical to CBF1 (Stockinger et al., 1997). The DREB1C cDNA contains a single open reading frame of 216 amino acids and encodes a putative protein with a predicted molecular mass of 24.3 kD (Figure 8A). The three DREB1A homologs have highly homologous amino acid sequence identity (Figure 8A, DREB1A and DREB1B, 86%; DREB1B and DREB1C, 86%; DREB1A and DREB1C, 87%). Moreover, the low-temperature-inducible expression of the *DREB1B* and the *DREB1C* genes was also similar to that of the *DREB1A* gene (data not shown).

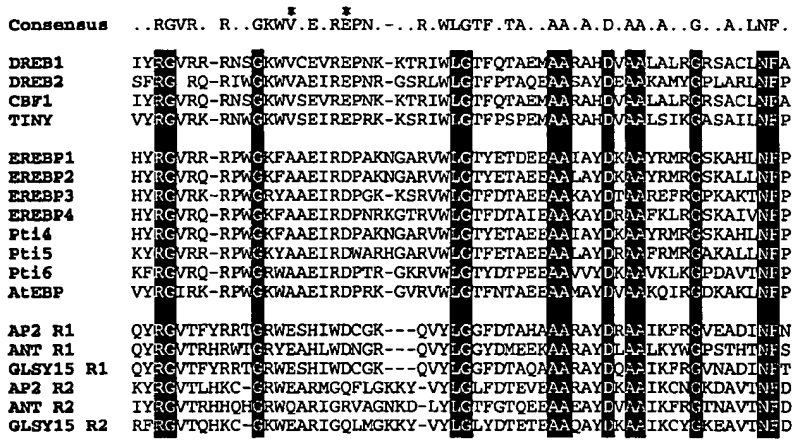

Figure 4. Comparison of Deduced Amino Acid Sequences of the DNA Binding Domains of DREB1A and DREB2A with Those of Other EREBP/AP2–Related Proteins.

The deduced amino acid sequences of DREB1A and DREB2A are compared with EREBP/AP2–related proteins, namely, Arabidopsis CBF1 (Stockinger et al., 1997), Arabidopsis TINY (Wilson et al., 1996), tobacco EREBP1 to EREBP4 (Ohme-Takagi and Shinshi, 1995), tomato Pti4 to Pti6 (Zhou et al., 1997), Arabidopsis AtEBP1 (AtEBP; Buttner and Singh, 1997), Arabidopsis AP2 (Jofuku et al., 1994), Arabidopsis AINTEGUMENTA (ANT; Klucher et al., 1996), and maize GLOSSY15 (GLSY15; Moose and Sisco, 1996). The black background represents perfectly conserved amino acid residues, and dashes indicate gaps introduced to maximize alignment. Consensus indicates the conserved amino acids in DREB1A and DREB2A. Asterisks represent different amino acids in the consensus between DRE binding proteins and GCC box binding proteins. R1 and R2 indicate repeated amino acid sequences of the EREBP/AP2 motif.

No DREB2A homolog was isolated by screening cDNA libraries by using the DREB2A cDNA as a probe. Arabidopsis cDNA libraries were then screened using the yeast one-hybrid system. We screened 7.6×10^6, 7.4×10^6, and 7.8×10^6 yeast transformants of the libraries prepared from 2-hr dehydrated, 24-hr cold-treated, and unstressed Arabidopsis rosette plants, respectively. We isolated cDNA clones encoding a DREB2A homolog from the library prepared from 2-hr dehydrated plants (named DREB2B; Figure 8B). The DREB2B cDNA contains a single open reading frame of 330 amino acids and encodes a putative protein with a predicted molecular mass of 37.1 kD (Figure 8B). DREB2A and DREB2B show sequence similarity (Figure 8B; 53.8%), especially in the N-terminal region. A serine- and threonine-rich region following the DNA binding domain and a glutamine-rich region in the C-terminal region were found in both the DREB2A and DREB2B proteins (Figure 8B). The dehydration-induced and high-salinity-induced expression of the *DREB2B* gene was similar to that of the *DREB2A* gene (data not shown). These observations indicate that the two *DREB2* genes are clearly different from the three *DREB1* genes.

DREB1A and DREB2A Proteins Transactivate the *rd29A* Promoter-*GUS* Fusion Gene in Leaf Protoplasts

To determine whether the DREB1A and DREB2A proteins are capable of transactivating DRE-dependent transcription in plant cells, we performed transactivation experiments using protoplasts prepared from Arabidopsis leaves. Protoplasts were cotransfected with a β-glucuronidase (*GUS*) reporter gene fused to the trimeric 71-bp fragments containing the DRE motif and the effector plasmid (Figure 9A). The effector plasmid consisted of the cauliflower mosaic virus (CaMV) 35S promoter fused to DREB1A or DREB2A cDNAs. The tobacco mosaic virus (TMV) Ω sequence was inserted upstream from these cDNAs to strengthen their translation efficiency. Coexpression of the DREB1A or DREB2A proteins in protoplasts transactivated the expression of the *GUS* reporter gene (Figure 9B). These results suggest that DREB1A and DREB2A proteins function as transcription activators involved in the cold- and dehydration-responsive expressions, respectively, of the *rd29A* gene.

Analysis of the in Vivo Roles of DREB1A and DREB2A in Expression of the *rd29A* Gene by Using Transgenic Plants

To analyze the effects of overproduction of DREB1A and DREB2A proteins on the expression of the *rd29A* gene, we generated transgenic plants in which DREB1A or DREB2A cDNAs were introduced to overproduce DREB proteins. Arabidopsis plants were transformed with binary vectors carrying fusions of the enhanced CaMV 35S promoter (Mituhara et al., 1996) and the DREB1A (35S:DREB1A) or DREB2A (35S:DREB2A) cDNAs in the sense orientation. The TMV Ω sequence (Gallie et al., 1987) was inserted upstream from

these cDNAs to strengthen their translation level. Eighteen and eight antibiotic-resistant Arabidopsis transformants for DREB1A and DREB2A, respectively, were generated by using a vacuum infiltration method (Bechtold et al., 1993). Transgenic plants of the T_2 generation were used for further analyses.

All of the 18 plants carrying the 35S:DREB1A transgene (the 35S:DREB1A plants) had dwarf phenotypes under normal growth conditions. The 35S:DREB1A plants showed variations in phenotypic changes in growth retardation that may have been due to the different levels of expression of the DREB1A transgenes for the position effect. Three different phenotypic changes in growth of the 35S:DREB1A plants were compared with wild-type plants (Figure 10). Three of the 18 35S:DREB1A plants, including the 35S:DREB1Aa plants, showed severe dwarf phenotypes, whereas the others revealed growth retardation as shown by the results with the 35S:DREB1Ab and 35S:DREB1Ac plants (Figure 10). In the severely dwarfed 35S:DREB1Aa plants, the DREB1A transcript accumulated to a high level under the unstressed control condition. The higher level of the DREB1A transcripts in the 35S:DREB1A plants caused the more severe dwarf phenotypes of the transgenic plants (Figure 11).

To analyze whether overproduction of the DREB1A protein caused the expression of the target gene in unstressed plants, we compared the expression of the *rd29A* gene in control plants carrying pBI121 vector (wild type) with that in the 35S:DREB1A plants. Transcription of the *rd29A* gene was low in the unstressed wild-type plants but high in the unstressed 35S:DREB1A plants. The level of the *rd29A* transcripts under the unstressed control condition was found to depend on the level of the DREB1A transcripts. Expression of the *rd29A* gene was induced by dehydration, high salt and cold stress, and ABA treatment in the 35S:DREB1A plants as well as in wild-type plants. However, the level of the *rd29A* transcripts in the 35S:DREB1A plants was higher than that in the wild-type plants, even under stressed conditions (Figure 11).

The transgenic plants carrying the 35S:DREB2A transgene (the 35S:DREB2A plants) showed little phenotypic change. However, 35S:DREB2Aa plants exhibited slight growth retardation (Figure 10). The level of *DREB2A* mRNA was higher in 35S:DREB2Aa plants than in the normal 35S:DREB2Ab plants (Figure 11). Expression of the *rd29A* gene in the 35S:DREB2Aa plants having little phenotypic change under unstressed conditions was slightly higher than in the normal 35S:DREB2Ab plants (Figure 11).

Freezing and Dehydration Tolerance of Transgenic Plants

The tolerance to freezing and dehydration of the transgenic plants was analyzed using the 35S:DREB1Ab and 35S:DREB1Ac plants grown in pots at 22°C for 3 weeks. When plants grown in pots were exposed to a temperature of −6°C for 2 days, returned to 22°C, and grown for 5 days,

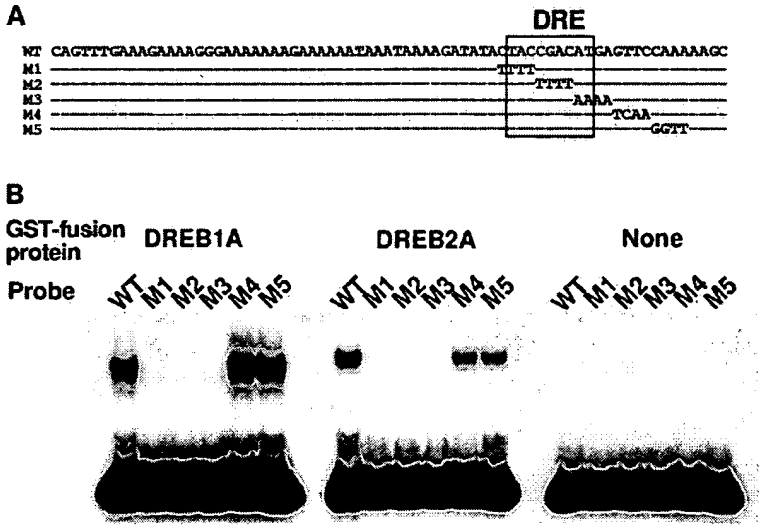

Figure 5. Characterization of the DNA Binding Affinity of the Recombinant DREB1A and DREB2A Proteins to the 71-bp Fragment (Positions −215 to −145) of the *rd29A* Promoter.

(A) Upper strand sequence of the 71-bp fragment of the *rd29A* promoter (WT) and its mutated fragments (M1 to M5) used as probes.
(B) Gel retardation assay of sequence-specific binding of the recombinant DREB1A and DREB2A proteins. The radioactive probes were incubated in the presence or absence (None) of the recombinant DREB1A or DREB2A proteins.

all of the wild-type plants died, whereas the 35S:DREB1Ab plants survived at high frequency (83.9% survival; Figure 12). The surviving plants continued to grow and flowered under unstressed conditions. Freezing tolerance was correlated with the level of expression of the stress-inducible genes under unstressed control conditions. The 35S:DREB1Ab plants with high-level expression of the target genes showed higher freezing tolerance than did the 35S:DREB1Ac plants with their low-level expression (35.7% survival; Figure 12).

To test whether the introduction of the DREB1A gene enhances tolerance to dehydration stress, for 2 weeks we did not water the wild-type and transgenic plants grown in pots (Figure 12). Although all of the wild-type plants died within 2 weeks, 42.9% of the 35S:DREB1Ab plants survived and continued to grow after rewatering. Drought tolerance was also dependent on the level of expression of the target genes in the 35S:DREB1A plants under unstressed conditions. The survival rate of the 35S:DREB1Ac plants was lower than that of the 35S:DREB1Ab plants (21.4% survival; Figure 12).

DISCUSSION

Using the yeast one-hybrid screening system, we identified two distinct cDNAs, DREB1A and DREB2A, encoding DNA binding proteins that specifically interact with the DRE sequence involved in dehydration-, high-salt-, and low-temperature-responsive gene expression. The DREB1A and

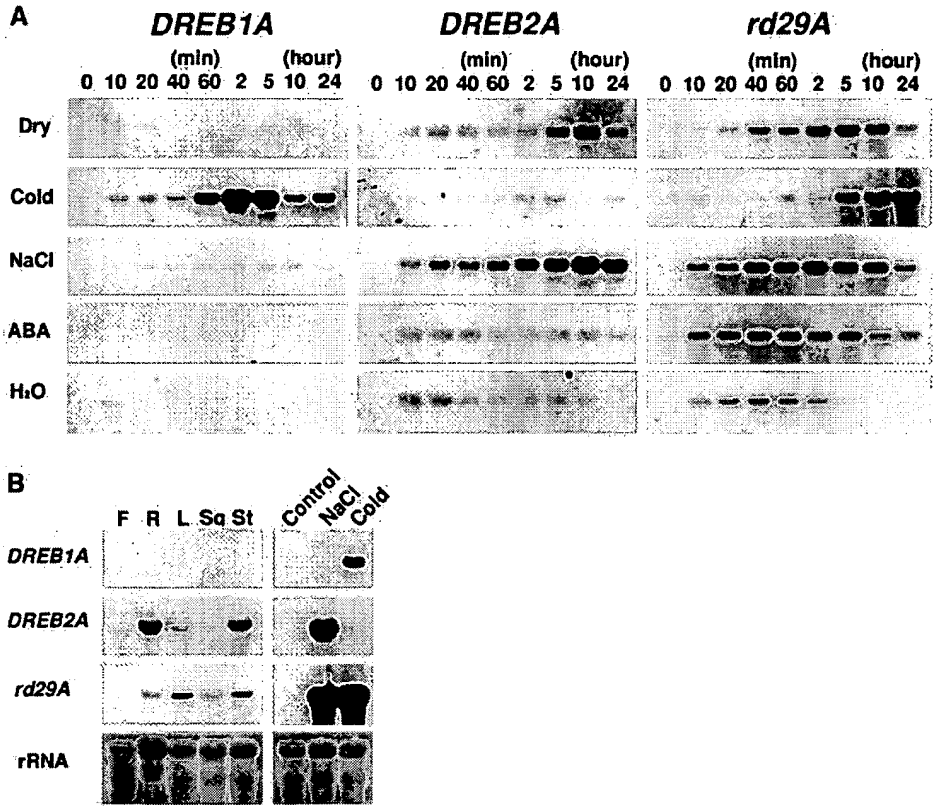

Figure 6. RNA Gel Blot Analysis of DREB1A and DREB2A Transcripts.

(A) Expression of the *DREB1A*, *DREB2A*, and *rd29A* genes in response to dehydration, low temperature, high salt, or ABA. Each lane was loaded with 20 μg of total RNA from 3-week-old unbolted Arabidopsis plants that had been dehydrated (Dry), transferred to and grown at 4°C (Cold), transferred from agar plates for hydroponic growth in 250 mM NaCl (NaCl), transferred from agar plates to hydroponic growth in 100 μM ABA (ABA), or transferred from agar plates to water (H_2O) for hydroponic growth, as described in Methods. The number above each lane indicates the number of minutes or hours after the initiation of treatment before isolation of RNA. RNA was analyzed by RNA gel blotting, with gene-specific probes from the 3′ flanking sequences of *DREB1A*, *DREB2A*, and *rd29A*.

(B) Expression of the *DREB1A*, *DREB2A*, and *rd29A* genes in a variety of organs of normally grown Arabidopsis. Each lane was loaded with 40 μg of total RNA prepared from flowers (F), roots (R), leaves (L), siliques (Sq), stems (St), whole plants (Control), whole plants treated with 250 mM NaCl for 5 hr (NaCl), and whole plants cold-treated at 4°C for 5 hr (Cold). rRNAs blotted on the membrane were visualized by staining with methylene blue.

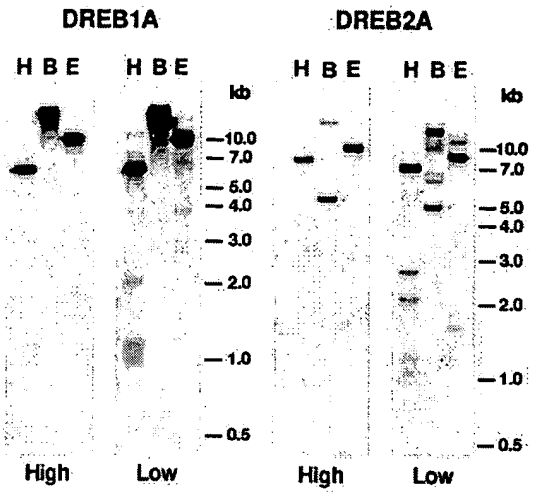

Figure 7. DNA Gel Blot Analysis of Genomic Sequences That Correspond to DREB1A and DREB2A cDNAs.

Genomic DNA was digested with HindIII (H), BamHI (B), and EcoRI (E). A full-length DREB1A or DREB2A cDNA was used as a probe. Filters were washed in either 0.5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.5% SDS at 50°C (low stringency; Low) or 0.1 × SSC and 0.1% SDS at 65°C (high stringency; High). Numbers at right are molecular length markers in kilobases.

DREB2A proteins were demonstrated to function as transcriptional activators for DRE-dependent transcription not only in yeast cells (Figure 2) but also in transactivation experiments using Arabidopsis leaf protoplasts (Figure 9). These results strongly suggest that both the DREB1A and DREB2A proteins are involved in DRE-dependent expression of the *rd29A* gene. However, they have no significant sequence identity, except for the conserved DNA binding domain, which suggests that they can be assigned to different classes. Two DREB1A-related cDNAs, DREB1B and DREB1C, and one DREB2A-related cDNA, DREB2B, were isolated by screening cDNA libraries prepared from dehydrated, cold-treated, and untreated Arabidopsis plants (Figure 8). These observations suggest that at least five distinct DRE binding proteins in two groups, DREB1 and DREB2, bind to the same target sequence, DRE, and are involved in the activation of the *rd29A* gene in response to dehydration, high-salt, and low-temperature stress.

The bacterially expressed DREB1A and DREB2A proteins specifically bound to the DRE sequence (Figure 5). Both the DREB1A and DREB2A proteins bound to the 71-bp DNA fragment with the DRE sequence but not to the 71-bp DNA fragments with base substitutions in the DRE sequence (M1, M2, and M3). In contrast, the fusion proteins bound to the 71-bp DNA fragments with base substitutions (M4 and M5) in the flanking sequence (Figure 5). These results indicate that the DRE sequence is the target sequence for DNA binding of the DREB1A and DREB2A proteins.

We have shown, using transgenic tobacco and Arabidopsis plants, that the DRE sequence is essential for the transcription of *rd29A* under conditions of drought, high salt, and low temperature (Yamaguchi-Shinozaki and Shinozaki, 1994). We used the same set of 71-bp fragments for the analyses of *cis*-acting elements. The 71-bp fragments with base substitutions (M1, M2, and M3) in the DRE sequence did not function in dehydration-induced expression, whereas the 71-bp fragments with base substitutions (M4 and M5) in the flanking sequence responded to dehydration stress in transgenic plants. These results coincide with the DNA

A

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DREB1A  MNSFSAFSEMFGSDYESSVSSGGDYIPTLASSCPKKPAGR   40
DREB1B  ******************-P--Q****C*T**T**********  37
DREB1C  ****************SFVSS****S*K**T**********  40

DREB1A  KKFRETRHPIYRGVRRRNSGKWVCEVREPNKKTRIWLGTF   80
DREB1B  ***************Q*******S*V***************  77
DREB1C  ***************Q*******C*L***************  80

DREB1A  QTAEMAARAHDVAALALRGRSACLNFADSAWRLRIPESTC  120
DREB1B  ***************L************************  117
DREB1C  ***************I************************  120

DREB1A  AKDIQKAAAEAALAFQDEMCDATT-DHGFDMEETLVEAIY  159
DREB1B  **D**********A****T*DT**TN**L*****M*****  157
DREB1C  **E**********N****M*HM**DA**L*****L*****  160

DREB1A  TAEQSENAFYMHDEAMFEMPSLLANMAEGMLLPLPSVQNN  199
DREB1B  *P***EG****DE*T*FG*PT**D*********P******  197
DREB1C  *P***QD****DE*A*LG*SS**D*********S******  201

DREB1A  HNHEVDGDDDDVSLWSY                         216
DREB1B  H*YDGE*-*G*******                         213
DREB1C  Y*FDVE*-*D*******                         217
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B

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DREB2A  MAVYDQSGDRNRTQIDTSRKRKSRSRGDGTTVAERLKRMK   40
DREB2B  ****E*T*----*E-Q-PK*****A*AG*L***D***K**   34

DREB2A  EYNETVE--EV--STK-KREVPAKGSKKGCMKGKGGPENS   75
DREB2B  ****I**ASA*KEGE*P******************D**     74

DREB2A  RCSFRGVRQRIWGKWVAEIREPNRGSRLWLGTFPTAQEAA  115
DREB2B  H*********************KI*T**********EK**  114

DREB2A  SAYDEAAKAMYGPLARLNFPRSDASEVTSTSSQSEVCTVE  155
DREB2B  *******T****S*******Q*VG**P*************  154

DREB2A  -TP-GC--VHVKTEDPDCESKPFSG--GVEPMYCLENGAE  189
DREB2B  NKAVV*GD*C**H**T****N***QILD*REES*GTRPDS  194

DREB2A  -EM-KRGVKAD-K-HWLSEFEHNYWSDILKEKEKQKEQGI  225
DREB2B  CTVGHQDMNSSLNYDL*L***QQ**GQV*Q****P*QEE-  233

DREB2A  VETCQQQQQDSLSVADYGWPNDVDQSHLDSSDMFDVDELL  265
DREB2B  -*EI*****EQ-QQQQLQ-*DLLTVADYGWPWSN*IVNDQ  270

DREB2A  RDLNGDDVFAGLNQDRYPGNSVANGSYRPESQQSGFDPLQ  305
DREB2B  TSWDPNEC*-DI*-E-LL*D-LNEPG-PHQ**D-Q-NHVN  303

DREB2A  SLNYGIPPFQLEGKDGNGFFDDLSYLDLEN             335
DREB2B  *GS*DLH*LH**PH**HE-*NQ**S**I--             330
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Figure 8. Comparison of the Deduced Amino Acid Sequences of the DREB1 and DREB2 Families.

Asterisks represent identical amino acid residues, and dashes indicate gaps introduced to maximize alignment. The underlined regions indicate the EREBP/AP2 DNA binding domains. A conserved Ser/Thr–rich region in DREB2A and DREB2B is indicated by a dashed underline.
(A) Comparison of DREB1A, DREB1B, and DREB1C.
(B) Comparison of DREB2A and DREB2B.

binding specificity of the two DREB proteins to the DRE sequence (Figure 5).

Both the DREB1A and DREB2A proteins contain a typical EREBP/AP2 DNA binding motif (Figure 4), which is found in tobacco EREBPs (Ohme-Takagi and Shinshi, 1995) and Arabidopsis AP2 (Jofuku et al., 1994). Recently, the EREBP/AP2 DNA binding motif also has been found in various plant regulatory genes, such as Arabidopsis *TINY* (Wilson et al., 1996), *CBF1* (Stockinger et al., 1997), *AtEBP* (Buttner and Singh, 1997), and *AINTEGUMENTA* (Elliott et al., 1996; Klucher et al., 1996), maize *Glossy15* (Moose and Sisco, 1996), and tomato *Pti5* (Zhou et al., 1997).

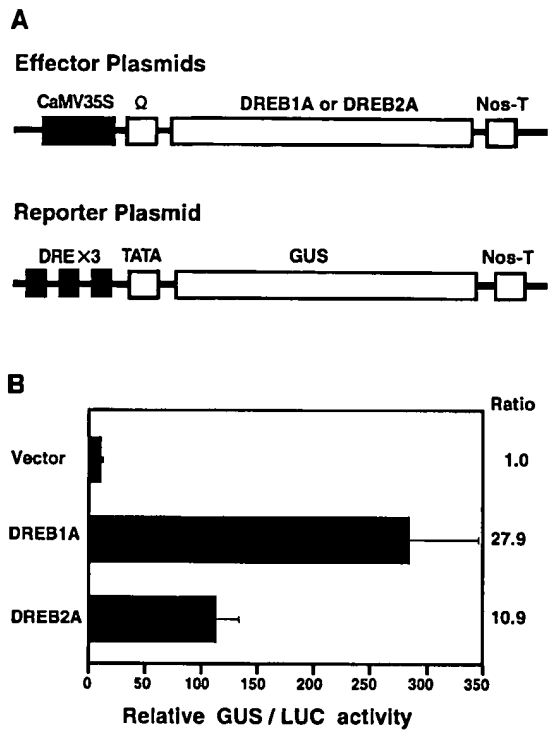

Figure 9. Transactivation of the *rd29A* Promoter–*GUS* Fusion Gene by DREB1A and DREB2A Proteins by Using Arabidopsis Protoplasts.

(A) Schematic diagram of the effector and reporter constructs used in cotransfection experiments. The effector constructs contain the CaMV 35S promoter and TMV Ω sequence (Gallie et al., 1987) fused to the DREB1A or DREB2A cDNA. Nos-T indicates the polyadenylation signal of the gene for nopaline synthetase. The reporter construct contains the 71-bp fragments of the *rd29A* promoter tandemly repeated three times (DRE × 3). The promoter was fused to the −61 *rd29A* minimal TATA promoter–*GUS* construct.

(B) Transactivation of the *rd29A* promoter–*GUS* fusion gene by the DREB1A and DREB2A proteins. The reporter gene was transfected with each effector plasmid or the vector as control treatments. To normalize for transfection efficiency, the CaMV 35S promoter–luciferase (LUC) plasmid was cotransfected in each experiment. Bars indicate the standard error of three replicates. Ratios indicate the multiplicities of expression compared with the value obtained with the pBI35SΩ vector.

These genes are divided into two classes based on the number of the EREBP/AP2 motifs. One class includes *AP2*, *AINTEGUMENTA*, and *Glossy15*, each of which encodes a protein containing two EREBP/AP2 motifs. The other class includes *EREBPs*, *TINY*, *CBF1*, *Pti5*, *AtEBP*, and *DREBs*, each of which encodes a protein with only one EREBP/AP2 motif. The *EREBPs*, *Pti5*, and *AtEBP* in the second class specifically bind to the GCC box sequence containing the core GCCGCC sequence, which is present in the promoter region of a large number of ethylene-inducible genes encoding pathogenesis-related proteins (Ohme-Takagi and Shinshi, 1995). The DREB1A, DREB2A, and CBF1 proteins specifically bind to the DRE/C repeat sequence containing the core sequence, PuCCGAC. These sequences resemble the GCC box and contain CCGNC as a common core sequence.

We compared the amino acid sequences of the DNA binding domains of the DREB1A, DREB2A, and CBF1 proteins and found consensus amino acids in their DNA binding domains (Figure 4, Consensus). All of the consensus amino acids are conserved in the DNA binding domains of *EREBPs*, *Pti5*, and *AtEBP*, except for the fourteenth valine (V) and nineteenth glutamate (E) in the binding domain of DREB1A and DREB2A (Figure 4). These two amino acids are also conserved in the DREB1A and DREB2A homologs (Figure 8). These conserved amino acids in the binding domains of DREB1A, DREB2A, and their homologs, including CBF1, may be important for binding specificity to the target sequence, PuCCGAC. The EREBP/AP2 motif contains an 18–amino acid core region that has been proposed to form an amphipathic α-helix; the latter could play a role in protein–protein interactions to facilitate DNA binding (Okamuro et al., 1997). The region of DREB1A and DREB2A proteins also theoretically is capable of forming the amphipathic α-helix structure.

Expression of *DREB1A*, *DREB1B* (*CBF1*), and *DREB1C* was strongly induced by low-temperature stress, whereas that of *DREB2A* and *DREB2B* was induced by dehydration and high-salt stress (Figure 6; Z.K. Shinwari, K. Nakashima, S. Miura, M. Kasuga, K. Yamaguchi-Shinozaki, and K. Shinozaki, unpublished data). The *CBF1* gene is identical to the *DREB1B* gene. We demonstrated that *DREB1B*/*CBF1* as well as *DREB1A* and *DREB1C* gene expression is induced by low temperature, whereas Stockinger et al. (1997) showed that the *CBF1* gene is constitutively expressed even under unstressed conditions. This difference may be due to differences in stress treatment or growth conditions of Arabidopsis plants. *rd29A* gene expression was induced by dehydration, high-salt, and low-temperature stress (Figure 6). These results suggest that the DREB1A-related proteins function in the DRE/C repeat–dependent expression of *rd29A* during low-temperature stress, whereas the DREB2A-related proteins are involved in the expression of *rd29A* during dehydration and high-salt stress. The expression of the *DREB1A* genes during low-temperature stress precedes that of the *rd29A* gene (Figure 6). Furthermore, the induction

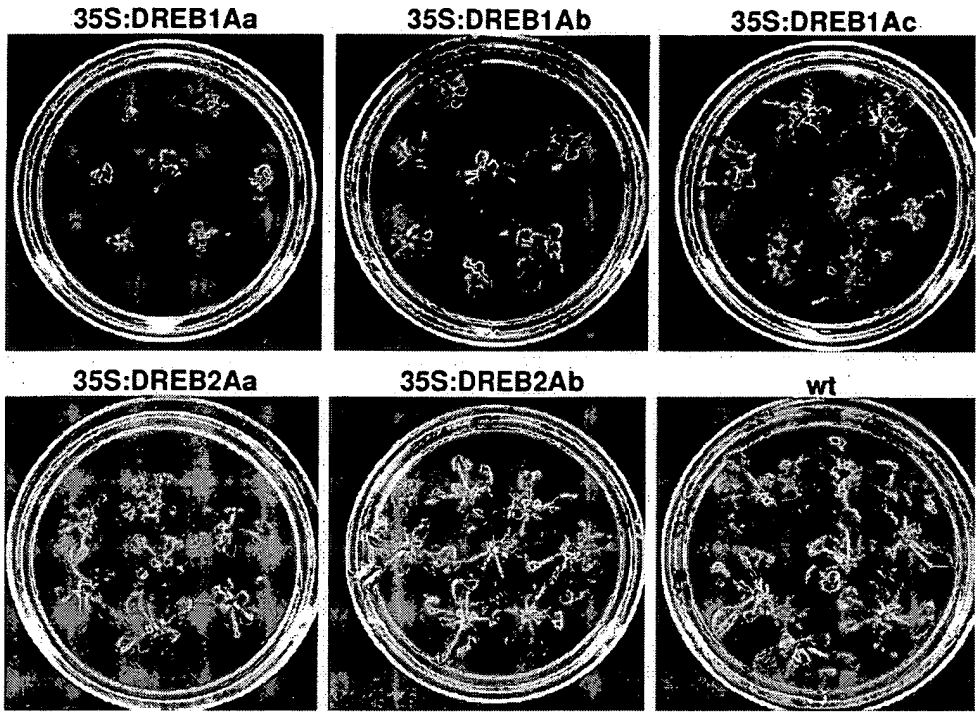

Figure 10. Effects of Overexpressing DREB1A and DREB2A cDNAs in Transgenic Plants.

Shown are 3-week-old seedlings carrying the 35S:DREB1A transgene with a variety of dwarf phenotypes (35S:DREB1Aa, 35S:DREB1Ab, and 35S:DREB1Ac), those carrying the 35S:DREB2A transgene with growth retardation (35S:DREB2Aa and 35S:DREB2Ab), and 3-week-old seedlings carrying pBI121 (wt).

of *rd29A* expression by high-salt and low-temperature stress was inhibited by half when cycloheximide was used (data not shown), whereas the induction of *rd29A* expression by exogenous ABA treatment was not inhibited (Yamaguchi-Shinozaki and Shinozaki, 1993). These results suggest that induction of the DREB proteins by these stresses is required for the expression of *rd29A*.

However, expression of the *rd29A* gene was induced rapidly by high salt (within 10 min) and dehydration (within 20 min), suggesting that the DREB2A-related transcription factors may be activated directly by these stresses. Indeed, we detected *DREB2A* mRNA in the unstressed control plants. However, we could not detect *DREB1A* mRNA in the unstressed plants (Figure 6B). These observations indicate that both induction and modification of the DREB2A transcription factors are needed for the transactivation of the *rd29A* gene under dehydration and high-salt stress conditions and that the induction of DREB1A transcription factors is important for the expression of *rd29A* under low-temperature stress conditions (Figure 13).

The above-mentioned hypothesis is supported by our analyses of transgenic plants that overexpressed the DREB1A or DREB2A cDNAs. The *rd29A* mRNA as well as the *DREB1A* mRNA accumulated in 35S:DREB1Aa transgenic plants that overexpressed the DREB1A cDNA and revealed a severe dwarf phenotype under unstressed growth conditions (Figures 10 and 11). The level of accumulated *DREB1A* mRNA correlated with the level of *rd29A* mRNA and the phenotypic changes of growth retardation of the transgenic plants (35S:DREB1Ab and 35S:DREB1Ac; Figures 10 and 11). The expression of the *rd17/cor47* gene, which is induced by dehydration and low temperature (Gilmour et al., 1992; Iwasaki et al., 1997), was also observed in the 35S:DREB1A plants under unstressed conditions (data not shown). The overproduction of the DREB1A-related proteins is enough to induce the expression of target genes. TINY has sequence similarity with DREBs and CBF1 (Figure 4). Ectopic overexpression of the TINY protein by the 35S promoter resulted in a semidominant dwarf phenotype (Wilson et al., 1996). This phenotype may be due to an effect similar to that causing overexpression of DREB1A in transgenic plants. The 35S:DREB1A transgenic plants revealed freezing and dehydration tolerance (Figure 12) as well as growth retardation. This may have been due to the overexpression of stress-inducible genes that are controlled by the DREB proteins under unstressed conditions. Overproduction of stress-related proteins is likely to make these transgenic plants more stress tolerant under normal growth

conditions, which may cause growth retardation of the plants. Independently, Jaglo-Ottosen et al. (1998) reported that CBF1 overexpression also enhances freezing tolerance.

In contrast, 35S:DREB2A transgenic plants that overexpressed the DREB2A cDNA revealed weak phenotypic changes in growth retardation experiments (35S:DREB2Aa; Figure 10). In 35S:DREB2A transgenic plants, *rd29A* mRNA did not accumulate significantly, although *DREB2A* mRNA accumulated even under unstressed conditions (Figure 11). Expression of the DREB2A protein is not sufficient for the induction of the *rd29A* gene. Modification, such as phosphorylation of the DREB2A protein, seems to be necessary for it to function in response to dehydration. The DREB2A and DREB2B proteins contain a conserved Ser/Thr–rich region adjacent to the EREBP/AP2 DNA binding domain, although the DREB1A-related proteins do not (Figure 8). We are investigating whether this Ser/Thr–rich region is phosphorylated under dehydration conditions.

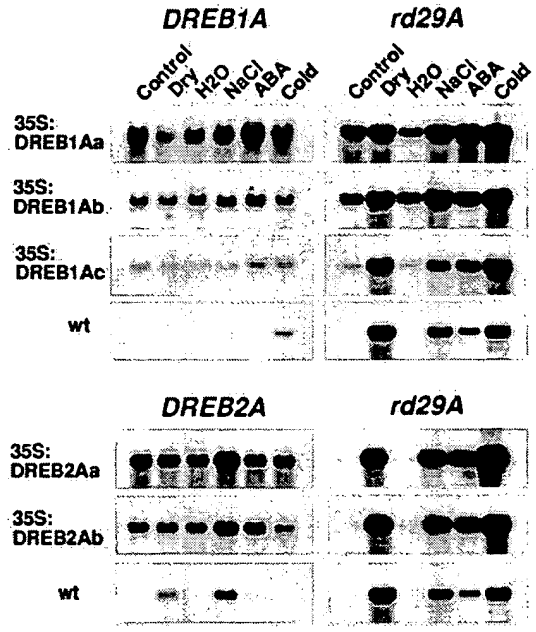

Figure 11. Expression Analyses of the *DREB1A*, *DREB2A*, and *rd29A* Genes in Transgenic Plants.

RNA gel blotting was conducted to measure the amount of *DREB1A*, *DREB2A*, or *rd29A* mRNA in transgenic Arabidopsis plants carrying the 35S:DREB1A transgene (35S:DREB1Aa, 35S:DREB1Ab, and 35S:DREB1Ac), those carrying the 35S:DREB2A transgene (35S:DREB2Aa and 35S:DREB2Ab), and those carrying pBI121 (wt). Transgenic plants were dehydrated (Dry), transferred from agar plates for hydroponic growth in water (H_2O), transferred from agar plates for hydroponic growth in 250 mM NaCl (NaCl), transferred from agar plates for hydroponic growth in 100 μM ABA (ABA), transferred to 4°C (Cold), and then treated for 5 hr under each condition or were untreated (Control). DNA fragments for the DREB1A and DREB2A cDNAs or the 3′ flanking region of *rd29A* were used as probes.

Figure 13 summarizes a model of the role of the two DREB proteins in the separation of two different signaling pathways under drought and cold stress conditions. Expression of DREB1A and its homologs is induced by low temperature, and the accumulated DREB1A homologs in turn transactivate the DRE-dependent gene expression of *rd29A*. Therefore, the expression of the *rd29A* gene is slower than that of DREB1A under low-temperature conditions. Overexpression of DREB1A in transgenic Arabidopsis activates the expression of the *rd29A* gene under normal unstressed conditions. Transgenic Arabidopsis plants overproducing DREB1A revealed abnormal stressed phenotypes. These observations indicate that the transcriptional activation of the *rd29A* gene is controlled directly by the induction of the DREB1A protein in Arabidopsis plants. In contrast, expression of DREB2A and its homolog is induced by drought and high salt; however, these genes are also expressed in unstressed control plants at low levels. The induced DREB2A and its homolog then transactivate DRE-dependent gene expression under drought and high-salt conditions.

The expression of the *rd29A* gene as well as the *DREB2A* gene is induced rapidly by drought and high-salt stress. In this case, the induction of DREB2A proteins alone is not sufficient for the induction of *rd29A* gene expression; the DREB2A protein probably requires modification (such as phosphorylation) for its function because overexpression of DREB2A had little effect on the expression of the *rd29A* gene under unstressed conditions (Figure 11). A stress signal is necessary for the modification of the DREB2A homologs to their active forms in the transcription of the *rd29A* gene under water-deficient conditions. Both the DREB1A and DREB2A families of proteins bind to the same *cis*-acting element, DRE, and activate the gene expression, but these two families of proteins function in different signal transduction pathways under low-temperature and dehydration stress.

METHODS

Plant Materials and Stress Treatments

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates for 3 weeks, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). Dehydration, high-salt, and cold stress treatments and treatment with abscisic acid (ABA) were performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). The plants were subjected to the stress treatments for various periods and then frozen in liquid nitrogen for further analyses.

Construction of Reporter Plasmids for Yeast One-Hybrid Screening

The 71-bp polymerase chain reaction fragment between positions −215 and −145 in the *rd29A* promoter, which contains a dehydration-responsive element (DRE) and HindIII sites at its 5′ and 3′ ends

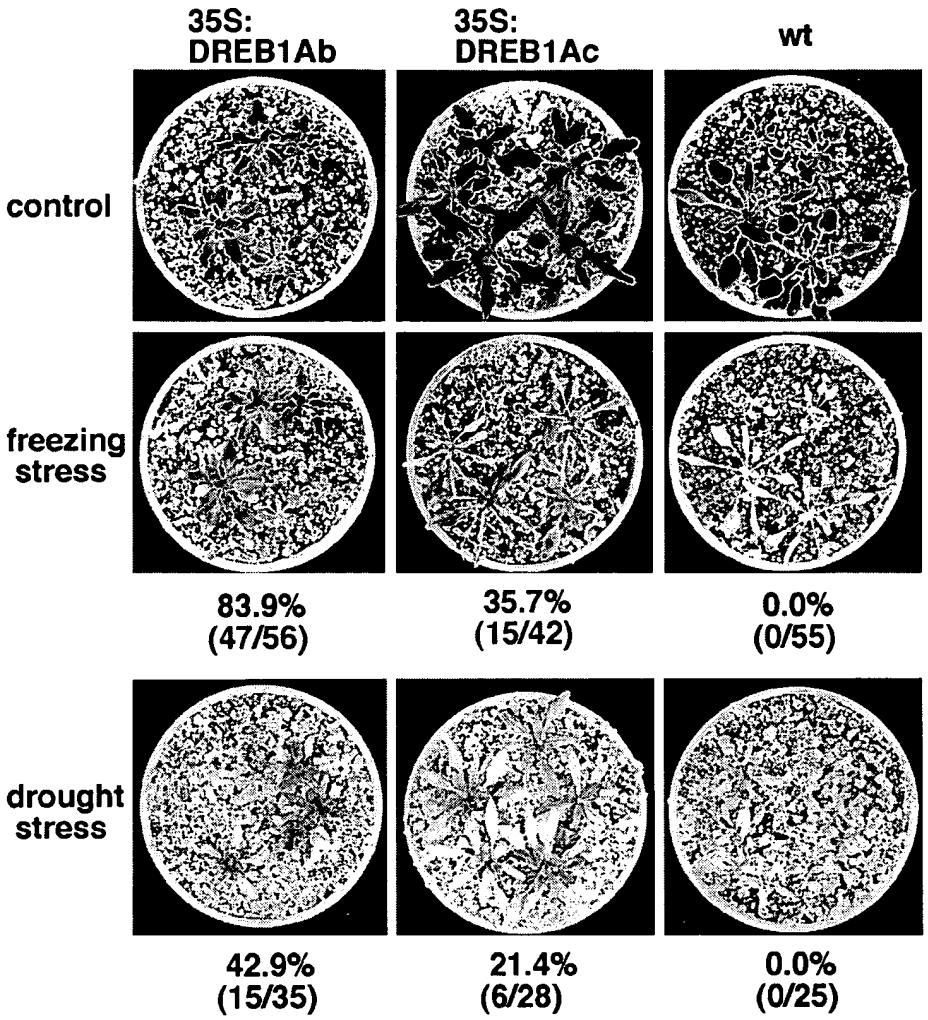

Figure 12. Freezing and Drought Tolerance of the 35S:DREB1Ab and 35S:DREB1Ac Transgenic Plants.

Control, 3-week-old plants growing under normal conditions; freezing stress, plants exposed to a temperature of −6°C for 2 days and returned to 22°C for 5 days; drought stress, water withheld from plants for 2 weeks. Percentages of surviving plants and numbers of surviving plants per total number of tested plants are indicated under the photographs. wt, wild type.

(Yamaguchi-Shinozaki and Shinozaki, 1994), was ligated into four tandemly repeated copies and then inserted into the HindIII site in the multicloning site (MCS) of the pBluescript II SK− (Stratagene, La Jolla, CA) vector. The fragment containing four tandem copies of the 71 bp was excised by EcoRI and HincII from the pBluescript II SK− vector and cloned into MCS upstream from the *HIS3* minimal promoter in the pHISi-1 expression vector, which had been digested with EcoRI and MluI (Clontech, Palo Alto, CA). The same fragment was excised by EcoRI and SalI from the pBluescript II SK− vector and cloned into MCS upstream from the *lacZ* minimal promoter in the pLacZi expression vector (Clontech), which had been digested with the same enzymes. Two kinds of expression plasmids were transformed simultaneously into yeast YM4271 strain (Figure 1). Yeast transformants containing the *HIS3* and *lacZ* reporter genes were obtained in selective medium plates (without His and Ura). The yeast transformant strains that could not grow under 10 mM 3-aminotriazole (3-AT) were used to screen the cDNA libraries.

Construction of Activation Domain–Tagged cDNA Libraries Derived from Dehydrated and Undehydrated Arabidopsis Rosette Plants

Twenty grams of whole rosette plants grown on GM agar plates for 3 weeks was used to prepare cDNA libraries. In the preparation of a cDNA library from dehydrated plants, we dehydrated harvested Arabidopsis plants at room temperature and 60% humidity under dim light for 2 hr and then froze them in liquid nitrogen. The weight of the plants decreased 22% after 2 hr of dehydration. In the preparation of a cDNA

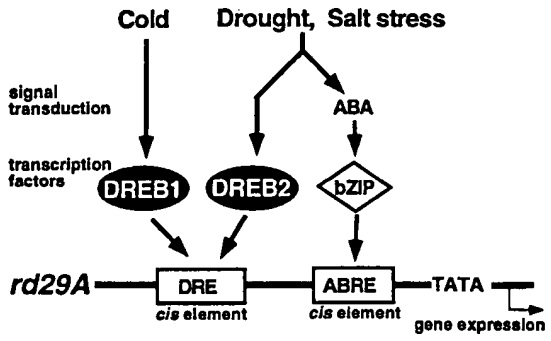

Figure 13. A Model for the Induction of *rd29A* Gene Expression under Dehydration, High-Salt, and Low-Temperature Conditions.

There are at least two independent signal transduction pathways—ABA independent and ABA responsive—between environmental stress and expression of the *rd29A* gene. The DRE functions in the ABA-independent pathway, and the ABA-responsive element (ABRE) is one of the *cis*-acting elements in the ABA-responsive induction of *rd29A*. Two independent families of DRE binding proteins, DREB1 and DREB2, function as *trans*-acting factors and separate two signal transduction pathways in response to cold, and drought and high-salinity stresses, respectively. bZIP, basic leucine zipper.

library from cold-treated plants, we transferred Arabidopsis plants to 4°C for 24 hr and harvested them. Total RNA, poly(A)$^+$ RNA, and cDNAs were prepared as described previously (Yamaguchi-Shinozaki et al., 1992). The cDNAs were ligated with the EcoRI-NotI-BamHI adapter fragment (Amersham) and then cloned into the EcoRI site in MCS downstream of the GAL4 activation domain in the pAD-GAL4 phagemid vector containing the *LEU2* reporter gene (Stratagene).

Yeast One-Hybrid Screening of Arabidopsis cDNA Libraries

Approximately 0.8×10^6, 1.2×10^6, and 1.5×10^6 yeast transformants were screened using cDNA libraries prepared from dehydrated, cold-treated, and unstressed Arabidopsis plants, respectively, according to the manufacturer's protocol (Clontech Matchmaker one-hybrid system). We obtained 203 positive colonies from selective medium plates (without His, Ura, and Leu but containing 10 mM 3-AT). Growth of these clones was examined at 30, 45, and 60 mM 3-AT. The β-galactosidase activities of the clones were then further analyzed. Finally, 41 clones, which grew normally on the 60 mM 3-AT plate and had β-galactosidase activity, were selected from the 203 positive clones. The cDNA of these 41 clones was isolated with a yeast DNA isolation system (Stratagene). The cDNA inserts were excised with EcoRI from the pAD-GAL4 plasmids and ligated into the pBluescript II SK− vector for sequencing.

Construction of Reporter Plasmids for the Transactivation Experiment with Yeast

To analyze transactivation activity of isolated cDNA clones, we fused three tandemly repeated copies of the wild-type or the mutated 71-bp fragment containing the DRE sequence to the MCS upstream from the *HIS3* minimal promoter in the pHISi-1 expression vector and the *lacZ* minimal promoter in the pLacZi expression vector, as shown in Figure 2. The DRE sequence TACCGACAT in the mutated 71-bp fragment was replaced with TATTTTCAT (Figure 5A, M2; Yamaguchi-Shinozaki and Shinozaki, 1994). These plasmids were transformed into the yeast YM4271 strain and used for the transactivation experiment with yeast (Figure 2).

Preparation of Glutathione *S*-Transferase Fusion Proteins and Gel Mobility Shift Assays

A 429-bp (119 to 547) fragment of the DREB1A cDNA and a 500-bp (167 to 666) fragment of the DREB2A cDNA were prepared by polymerase chain reaction and cloned into the EcoRI-SalI sites of the pGEX-4T-1 vector. The primer sets used for the amplification of the DREB1A and DREB2A cDNA fragments were 5′-CAGAGAATTCCGGATCCCAATGAACTCATTTTCTGCT-3′ and 5′-CCGCACTCGAGGTCGACCGTCGCATCACACATCTC-3′ and 5′-GATCCGAATTCATGGCAGTTTATGATCAGAGTGG-3′ and 5′-CAGCACTCGAGGTCGACGGATCCTCTGTTTTCAC-3′, respectively. The recombinant pGEX-4T-1 plasmids (Pharmacia) were transformed to *Escherichia coli* Blue XL-1. Production and purification of the glutathione *S*-transferase (GST) fusion proteins were performed as described previously (Urao et al., 1993). The 71-bp fragments containing DRE of the *rd29A* promoter with or without base substitutions were labeled with a ^{32}P-dCTP, as described previously. Gel mobility shift assays were conducted as described previously (Urao et al., 1993).

DNA and RNA Gel Blot Analyses

DNA gel blot hybridization and RNA gel blot hybridization were performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Transactivation Experiments with Protoplasts

Effector plasmids used in the transient transactivation experiment were constructed with DNA fragments containing the *DREB1A* or *DREB2A* coding regions that were cloned into polylinker sites of the plant expression vector pBI35SΩ, which was derived from pBI221 (Clontech). The pBI35SΩ vector was constructed as described previously (Abe et al., 1997). To construct 35S–Ω–DREB1A and 35S–Ω–DREB2A, we cloned the NotI fragment containing the coding region of DREB1A or DREB2A cDNA into the NotI site of the pBI35SΩ vector. To construct a reporter plasmid, we replaced the 35S promoter of pBI221 (Yamaguchi-Shinozaki and Shinozaki, 1994) with the *rd29A* minimal TATA promoter, and we then ligated the 71-bp fragment of the *rd29A* promoter into the HindIII site located upstream from the *rd29A* minimal TATA promoter with three tandem copies.

Isolation of Arabidopsis mesophyll protoplasts and polyethylene glycol–mediated DNA transfection were performed as described previously (Abel and Theologis, 1994). β-Glucuronidase (GUS) activity was measured as picomoles of product formed per minute per milligram of protein by using the standard protocol (Jefferson et al., 1986). Luciferase assays were performed using a PicaGene luciferase assay kit (Toyo-Ink, Tokyo, Japan), according to the manufacturer's instructions. Protein concentration was determined by the Bradford method (Bio-Rad).

Transgenic Plants Overexpressing the DREB cDNAs

Plasmids used in transformation of Arabidopsis were constructed with DREB1A or DREB2A full-length cDNA that was cloned into a polylinker site of a binary vector, pBI2113Not, which was derived from pBI2113 (Mituhara et al., 1996) in the sense orientation. For the construction of the pBI2113Not vector, pBI2113 was digested with SmaI and SacI to delete the GUS coding region and ligated with a SmaI–NotI–SacI polylinker (Takara, Tokyo, Japan). To construct the 35S:DREB1A and 35S:DREB2A plasmids, the EcoRV-SmaI fragment of the DREB1A cDNA and the SacI-EcoRV fragment of the DREB2A cDNA were cloned into the SacI-SmaI or SmaI site of the pBI2113Not vector, respectively. The constructs were introduced into *Agrobacterium tumefaciens* C58, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Arabidopsis plants used for transformation were grown in 8-cm pots filled with soil under continuous illumination at ~2500 lux at 22°C for 6 weeks. Plants were transformed by the vacuum infiltration method, as described by Bechtold et al. (1993).

Freezing and Drought Stress Tolerance of Transgenic Plants

Plants were grown in 9-cm pots filled with a 1:1 mixture of perlite/vermiculite. They were grown under continuous illumination of ~2500 lux at 22°C. Three-week-old plants were exposed to freezing and drought stress. Freezing stress was conducted by exposure of plants to a temperature of −6°C for 2 days and returned to 22°C for 5 days. Drought stress was conducted by withholding water for 2 weeks.

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REVIEW

Molecular Studies on Stress-Responsive Gene Expression in *Arabidopsis* and Improvement of Stress Tolerance in Crop Plants by Regulon Biotechnology

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Abstract

Molecular studies have shown that several genes with various functions are induced by environmental stresses such as drought, high-salinity and low temperature in plants. Most of the dehydration responsive genes are induced by the plant hormone abscisic acid (ABA), but others are not. Expression analyses of dehydration-responsive genes have provided at least four independent regulatory systems (regulons) for gene expression in a model plant *Arabidopsis thaliana*. The *cis*-acting elements in the promoters of some genes that have a typical stress-inducible expression profile and the transcription factors that affect the expression of these genes have been analyzed. Transcription factors that bind to a DRE/CRT (dehydration-responsive element / C-repeat) *cis*-acting element were isolated and termed DREB1/CBF (DRE-binding protein 1/ C-repeat binding factor) and DREB2 (DRE-binding protein 2). Overexpression of DREB1/CBF in transgenic *Arabidopsis* plants increased tolerance to freezing, drought and high salt concentrations. The *DREB1/CBF* genes have been successfully used to improve abiotic stress tolerance in a number of different crop plants. Studies on the other transcription factors associated with stress response are in progress. We collaborate with many research groups to improve stress tolerant crop plants utilizing regulon biotechnology. We hope the results of these collaborative studies will contribute to the sustainable food production in developing countries and help to prevent the global-scale environmental damage.

Discipline: Biotechnology
Additional key words: DREB1, environmental stress, transcription factors, transgenic plants

Introduction

As plants are sessile organisms, they are directly exposed to environmental stresses such as drought, high salinity and low temperature. Plants respond to environmental stress, and the transduced signals cause expression of numerous genes associated with stress tolerance. A number of genes have been described that respond to environmental stresses such as drought, high salinity and low temperature in plants[4,15,33,47,48,60].

We isolated more than 60 independent cDNAs for dehydration inducible genes using molecular techniques such as differential screening in a model plant *Arabidopsis thaliana*[33,47,48]. Recently, 299 drought-inducible genes, 54 cold-inducible genes, and 213 high-salinity-stress-inducible genes were identified using a cDNA microarray containing around 7,000 independent *Arabidopsis* full-length cDNA groups[46,48]. Functions of their gene products have been predicted from sequence homology with known proteins. Genes induced during dehydration stress conditions are thought to function not only in protecting cells from dehydration by the production of important metabolic proteins (functional proteins) but also in the regulation of genes for signal transduction in the dehydration stress response (regulatory proteins). The functional proteins contain water channel proteins, chaperons, proteases, LEA (Late Embryogenesis Abundant) proteins, and enzymes for the synthesis of osmoprotectants (compatible solutes; sugars, proline, etc.). The regulatory proteins contain transcription factors, protein kinases, and enzymes for phosphoinositide (PI) turn-

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over, and enzymes for the synthesis of the plant hormone abscisic acid (ABA). So far, various kinds of functional proteins such as enzymes for the synthesis of osmoprotectants were overexpressed in plants to improve the stress tolerance[5,59]. However, it seems that the engineering of one enzyme is not enough as many kinds of stress responses are necessary for plants to survive in severe stress conditions.

In plants, one transcription factor can control the expression of many target genes through the specific binding of the transcription factor to the *cis*-acting element in the promoters of the target genes. Such kind of a transcription unit is called a "regulon". Northern analysis of dehydration-inducible genes revealed that there appear to be at least four independent regulons in *Arabidopsis* (Fig. 1). They are (1) DREB regulon, (2) NAC (NAM, ATAF1, 2, and CUC2) and ZF-HD (zinc-finger homeodomein) regulon, (3) AREB/ABF (ABA-responsive element binding protein / ABA-responsive element binding factor) regulon, and (4) MYC (myelocytomatosis oncogene) and MYB (myeloblastosis oncogene) regulon. The DREB regulon and the NAC and ZF-HD regulon are ABA-independent. The AREB/ABF regulon and the MYC and MYB regulon are ABA-dependent. Regulon biotechnology, by controlling the expression of the regulon system, is expected to improve the tolerance against stresses in plants.

DREB regulon involved in ABA-independent gene expression

1. Isolation of DREB1/CBF regulon and DREB2 regulon

The promoter of an *Arabidopsis* drought-, high-salinity- and cold-inducible gene *RD29A* (*responsive to dehydration 29A*) encoding a LEA-like protein has been

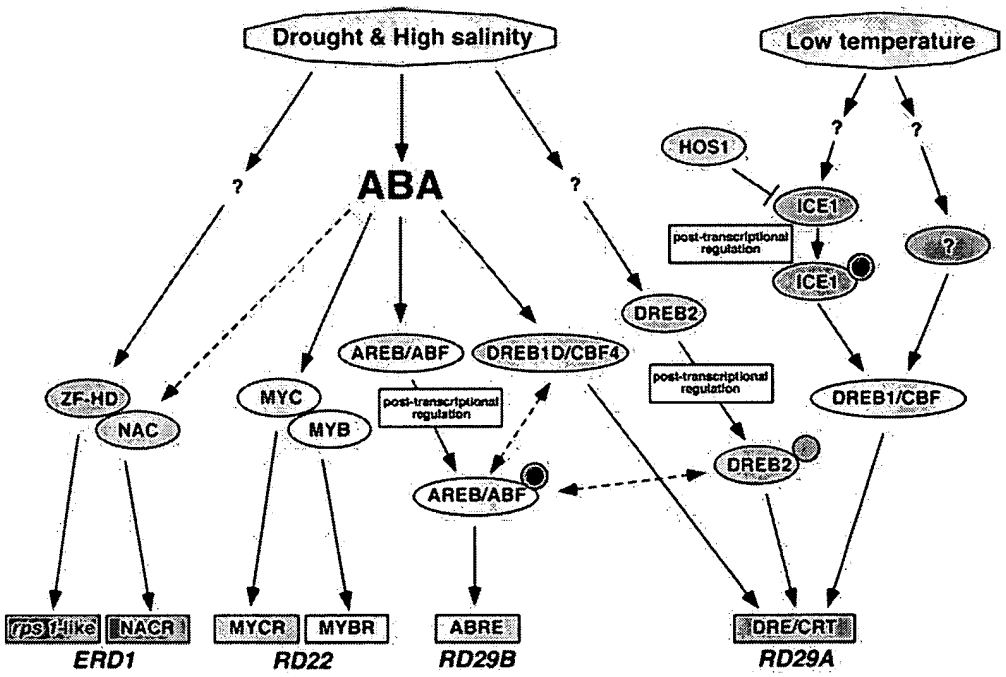

Fig. 1. Regulatory network of gene expression in response to drought, high salinity and cold stresses: specificity and crosstalk of gene networks

Cis-acting elements that are involved in stress-responsive transcription are shown in boxes. Transcription factors that control stress-inducible gene expression are shown in circles or ovals. Small circles indicate the modification of transcription factors in response to stress signals for their activation, such as phosphorylation. Dotted lines indicate possible regulation. Double arrow lines indicate possible cross talk. ABF: ABRE-binding factor, ABRE: ABA-responsive element, AREB: ABRE-binding protein, CBF: C-repeat-binding factor, CRT: C-repeat, DRE: dehydration-responsive element, DREB: DRE-binding protein, *ERD*: early responsive to dehydration, ICE: inducer of CBF expression, MYBR: MYB recognition site, MYCR: MYC recognition site, NACR: NAC recognition site, *RD*: responsive to dehydration, ZF-HD: zinc finger homeodomain protein.

found to contain two major *cis*-acting elements, the ABA-responsive element (ABRE) and the dehydration-responsive element (DRE)/C-repeat (CRT), that are involved in stress-inducible gene expression[56]. DRE/CRT (CCGAC) is a *cis*-acting element that functions in ABA-independent gene expression in response to abiotic stress (Fig. 1). Transcription factors belonging to the AP2/ERF (APETALA2 / ethylene-responsive element binding factor) family that bind to DRE/CRT have been isolated and termed DREB1/CBF and DREB2[10,26,50]. The conserved DNA-binding motif of DREB1/CBF and DREB2 is A/GCCGAC[42]. The *DREB1/CBF* genes are quickly and transiently induced by cold stress, and their products activate the expression of target stress-inducible genes. The *DREB2* genes are induced by dehydration, leading to the expression of various genes that are involved in drought-stress tolerance[26].

2. Improved stress tolerance of transgenic plants overexpressing *DREB1/CBF*

Overexpression of *DREB1A/CBF3* in transgenic *Arabidopsis* plants showed increased tolerance to freezing, drought and high salt concentrations[17,19,26], suggesting that the DREB1A/CBF3 proteins function without modification of the proteins in the development of stress tolerance. Many candidates for the DREB1A/CBF3 target genes have been identified using microarray[9,30,45]. Most of these target genes contain DRE- or DRE-related CCGAC core motif sequences in their promoter regions. We analyzed the expression of these candidate genes using RNA gel blot and identified more than 40 genes as the DREB1A downstream genes. Many of the products of these genes were proteins known to function against stress and were probably responsible for the stress tolerance of the transgenic plants. The downstream genes also included genes for transcription factors involved in further regulation of signal transduction and gene expression in response to stress.

The overexpression of the *DREB1/CBF* gene results in multiple biochemical changes associated with cold acclimation[11]: *DREB1A/CBF3*-expressing plants had elevated levels of proline (Pro) and total soluble sugars, including sucrose, raffinose, glucose, and fructose. Plants overexpressing *DREB1A/CBF3* also had elevated *P5CS* (for delta(1)-pyrroline-5-carboxylate synthase) transcript levels suggesting that the increase in Pro levels resulted, at least in part, from increased expression of the key Pro biosynthetic enzyme P5CS. These results lead us to propose that DREB1A/CBF3 integrates the activation of multiple components of the cold acclimation response.

Dwarfism is observed in transgenic *Arabidopsis* overexpressing *DREB1A/CBF3*, *DREB1B/CBF1*, *DREB1C/CBF2* or *DREB1D/CBF4*[11,12,19,26]. The development of dwarf phenotypes was also found in transgenic tomato overexpressing *Arabidopsis DREB1B/CBF1*, and it was prevented by exogenous application of gibberellin (GA)[14]. These suggest that an inhibition of GA biosynthesis is a function common to the *DREB1/CBF* genes. However, microarray analysis did not detect the changes in transcript levels of GA-related genes in transgenic *Arabidopsis* overexpressing *DREB1A/CBF3*, *DREB1B/CBF1*, or *DREB1C/CBF2*[9]. Recently, DREB1F is reported to be involved in the regulation of GA biosynthesis and stress tolerance[29]. It is not clear yet whether other DREB1/CBF proteins are related to GA synthesis or not.

In contrast to the *DREB1/CBF* genes, overexpression of *DREB2* in transgenic plants does not improve stress tolerance, suggesting that DREB2 proteins require posttranslational activation[26]. The DREB2 protein is expressed under normal growth conditions and is activated in the early stage of the osmotic stress response through posttranslational modification (Fig. 1).

3. Regulation of the expression of DREB1/CBF regulon

The *ICE1 (inducer of CBF expression 1)* gene was identified through the map-based cloning of the *Arabidopsis ice1* mutation, which affected the expression of the *DREB1A/CBF3* promoter-*LUC* (*luciferase*) transgene[6]. *ICE1* encodes a MYC-type bHLH (basic helix-loop-helix) transcription factor that regulates the expression of *DREB1A/CBF3* but not of other *DREB1/CBF* genes (Fig. 1). Overexpression of *ICE1* in transgenic plants resulted in improved freezing tolerance, supporting an important role for ICE1 in the cold-stress response. Molecular analysis of the *DREB1C/CBF2* promoter has identified multiple *cis*-acting elements that are involved in cold-inducible gene expression[57] (Imura et al., unpublished data). The DNA-binding protein has been cloned and shown to be a MYC-type bHLH transcription factor that is different from ICE1 (Imura et al., unpublished data). These results suggest the redundant involvement of MYC-type bHLH transcription factors in the up-regulation of the *DREB1/CBF* genes. A cold signal is necessary for the activation of the ICE proteins but the mechanism of this signal remains to be solved. Analysis of the *cbf2* mutant, in which the *DREB1C/CBF2* gene was disrupted, indicated that DREB1C/CBF2 is a negative regulator of *DREB1A/CBF3* and *DREB1B/CBF1* expression and plays a central role in stress tolerance in *Arabidopsis*[35]. These data suggest that the regulation of the expression of the *DREB1/CBF* genes might be more complex than previously thought.

NAC and ZF-HD regulon involved in ABA-independent gene expression

The *ERD1* (*early responsive to dehydration 1*) gene encoding a Clp (caseinolytic protease) protease regulatory subunit responds to dehydration and high salinity before the accumulation of ABA, suggesting the existence of an ABA-independent pathway in the dehydration stress response[31]. Analysis of the *ERD1* promoter identified two novel *cis*-acting elements that are involved in induction by dehydration stress[49]. Base substitution analysis showed that a 14-bp *rps1*-like region (CACTAAATTGTCAC) and a CATGTG motif are necessary for the induction of the *ERD1* gene in dehydrated plants (Fig. 1). Recently, we isolated three cDNA clones encoding proteins that bind to the 63-bp promoter region of *ERD1*, which contains the CATGTG motif[52] (Fig. 1). These three cDNA clones encode proteins which belong to the NAC transcription factor family including RD26. Microarray analysis of transgenic plants overexpressing the NAC genes revealed that several drought inducible genes were up-regulated in the transgenic plants, and the plants showed significantly increased drought tolerance. However, *ERD1* was not up-regulated in the transgenic plants. We recently isolated zinc-finger homeodomain (ZF-HD) transcription factors containing a homeodomain that can bind to the *rps1* site 1-like sequence using the yeast one-hybrid system. Overexpression of both NAC and ZF-HD proteins activated the expression of *ERD1* under unstressed normal growth conditions in the transgenic *Arabidopsis* plants.

AREB/ABF regulon involved in ABA-dependent gene expression

ABRE (ABA-responsive elements: ACGTGG/TC) is a major *cis*-acting element in ABA-responsive gene expression (Fig. 1). Two ABRE motifs are important in the ABA-responsive expression of the *Arabidopsis* gene *RD29B* encoding a LEA-like protein[53]. The bZIP (basic leucine zipper) transcription factors ABRE-binding protein (AREB)/ABRE-binding factor (ABF) can bind to ABRE and activate ABA-dependent gene expression[7,53]. Activation of the AREB1 and AREB2 proteins has been shown to require an ABA-mediated modification[53], which is probably ABA-dependent phosphorylation (Fig. 1). Overexpression of ABF3 or AREB2/ABF4 caused ABA hypersensitivity, reduced transpiration rate and enhanced drought tolerance of the transgenic plants[18]. The AREB1/ABF2 is reported to be an essential component of glucose signaling and its overexpression affects multiple stress tolerance including drought, salt and heat[22].

MYC and MYB regulon involved in ABA-dependent gene expression

The induction of the *Arabidopsis* drought-inducible gene *RD22* encoding a protein having a homology to an unidentified seed protein is mediated by ABA, and this gene requires protein biosynthesis for its ABA-dependent expression[1]. A MYC transcription factor, AtMYC2 (*Arabidopsis thaliana* MYC 2), and a MYB transcription factor, AtMYB2 (*Arabidopsis thaliana* MYB 2), have been shown to bind *cis*-elements, MYCR (MYC-recognition site: CANNTG) and MYBR (MYB-recognition site: C/TAACNA/G) in the *RD22* promoter and cooperatively activate *RD22*[1] (Fig. 1). These MYC and MYB proteins are synthesized after the accumulation of endogenous ABA, indicating that their role is in a late stage of the stress responses. Microarray analysis of MYC- and MYB-overexpressing transgenic plants revealed target genes for MYC and MYB, such as the alcohol dehydrogenase gene and ABA- or jasmonic-acid (JA)-inducible genes[2]. Overexpression of both AtMYC2 and AtMYB2 not only caused an ABA-hypersensitive phenotype but also improved the osmotic-stress tolerance of the transgenic plants[2].

Recently, AtMYC2 transcription factors function as members of a MYC-based regulatory system conserved in dicotyledonous plants with a key role in JA-induced defense gene activation[3,28]. These reports highlight the crosstalk between biotic stress signaling and abiotic stress signaling.

Crosstalk between the DREB regulon and the other regulons

Many drought- and cold-inducible genes contain both DRE/CRT and ABRE motifs in their promoters. These *cis*-acting elements are thought to function independently. However, precise analysis of these *cis*-acting elements in the *RD29A* gene expression revealed that DRE/CRT functions cooperatively with ABRE as a coupling element in ABA-responsive gene expression in response to drought stress[34]. This indicates that there are interactions between the DREB regulon and the AREB/ABF regulon (Fig. 1).

Recently, an osmotic-stress inducible *CBF4/DREB1D* gene has been identified[12]. Genes of the *DREB1/CBF* family are mainly induced by cold stress, but the drought-inducible gene *CBF4/DREB1D* functions to provide crosstalk between DREB2 and DREB1/CBF regulatory systems. The drought-inducible expres-

sion of *CBF4/DREB1D* is controlled by ABA-dependent pathways, suggesting that CBF4/DREB1D may function in the slow response to drought that relies on the accumulation of ABA (Fig. 1). Moreover, ABA induces the *DREB1/CBF* gene transcription and subsequent induction of cold-regulated genes via the DRE/CRT promoter element[24]. A maize DRE-binding protein, DBF1, has been shown to function as a transcriptional activator of the *rab17 (responsive to abscisic acid 17)* promoter by ABA[23]. This also suggests the existence in some plants of an ABA-dependent pathway for the regulation of stress-inducible genes that involves DRE/CRT.

Gene expression in recovery process from abiotic stress in *Arabidopsis*

Microarray analysis has revealed many genes that respond to rehydration after drought stress, indicating their involvement in the process of recovery from abiotic stress[36]. The products of these genes are thought to function not only in recovery from stress but also in cell growth and elongation. The expression and function of the rehydration-inducible *ERD5* gene encoding a proline dehydrogenase (ProDH) gene has been precisely analyzed. This gene is involved in the degradation of the proline that accumulates during dehydration[32]. Promoter analysis of the *ProDH* gene revealed an important *cis*-acting element, ACTCAT, that is involved in rehydration-inducible gene expression[43]. Many rehydration-inducible gene promoters contain the ACTCAT motif. Recently we showed that the ATB2 subgroup bZIP proteins functions as transcriptional activators in hypoosmolarity-responsive expression of the *ProDH* gene in *Arabidopsis*[44]. The molecular information in the process of recovery from abiotic stress may allow us to improve the resilient plants.

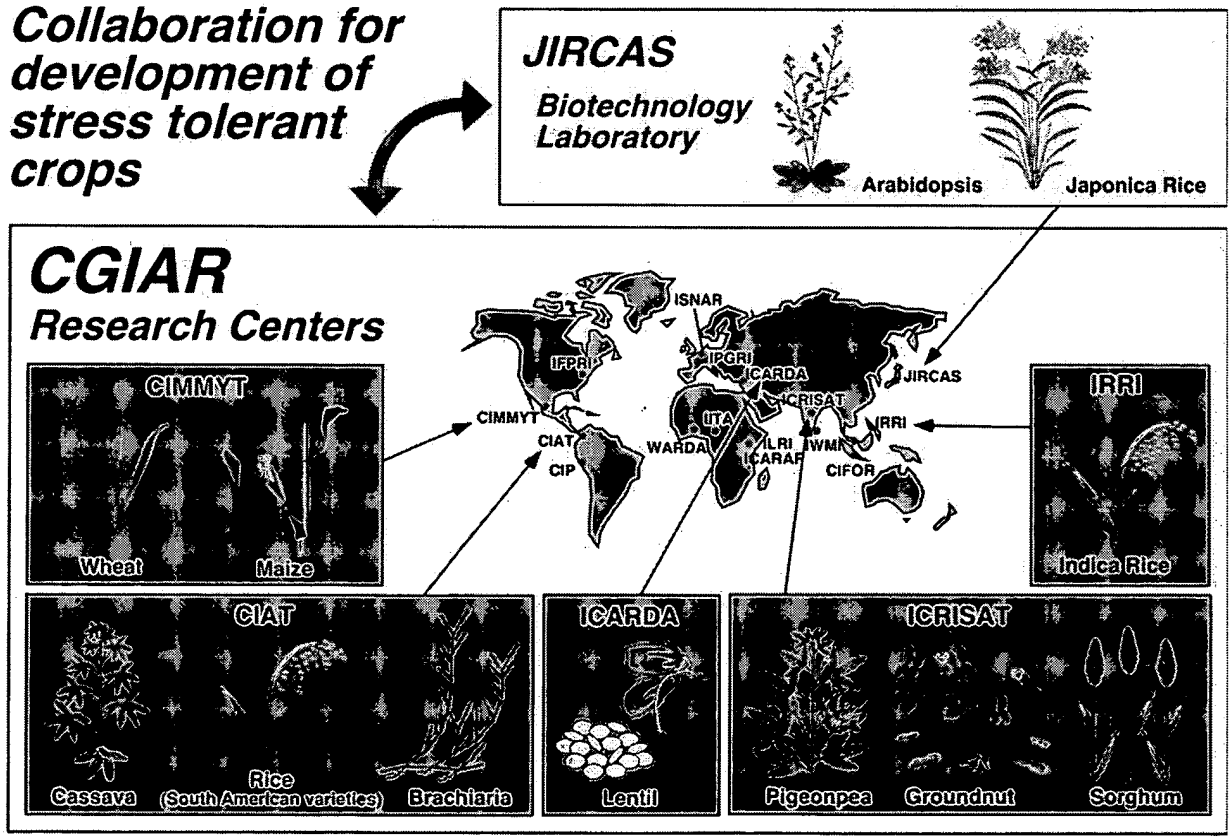

Fig. 2. Collaboration for development of stress tolerant crops

CGIAR: Consultative Group on International Agricultural Research, CIAT: International Center for Tropical Agriculture (Colombia), CIMMYT: International Maize and Wheat Improvement Center (Mexico), ICARDA: International Center for Agricultural Research in the Dry Areas (Syrian Arab Republic), ICRISAT: International Crops Research Institute for the Semi-Arid Tropics (India), IRRI: International Rice Research Institute (The Philippines), JIRCAS: Japan International Research Center for Agricultural Sciences (Japan).

Table 1. Abiotic stress tolerance by regulon biotechnology

Transcription factor	Type	Gene source	Transgenic Species	Promoter	Tolerance	Reference
DREB1A/CBF3	AP2/ERF	*Arabidopsis*	*Arabidopsis*	*35S, RD29A*	Freezing, salt, and drought	11, 19, 26
			Wheat	*RD29A*	Drought	39
			Tobacco	*35S, RD29A*	Freezing and drought	20
			Brassica napus	*35S*	Freezing and drought	16, 59
DREB1B/CBF1	AP2/ERF	*Arabidopsis*	*Arabidopsis*	*35S*	Freezing	11, 17
			Tomato	*35S, HVA22*	Drought, chilling, and oxidative stress	13, 14, 25
			Strawberry	*35S*	Freezing	37
			Brassica napus	*35S*	Freezing and drought	16, 59
DREB1C/CBF2	AP2/ERF	*Arabidopsis*	*Brassica napus*	*35S*	Freezing and drought	16, 59
DREB1D/CBF4	AP2/ERF	*Arabidopsis*	*Arabidopsis*	*35S*	Freezing and drought	12
DREB1F/DDF1	AP2/ERF	*Arabidopsis*	*Arabidopsis*	*35S*	High salinity	29
ZmDREB1	AP2/ERF	Maize	*Arabidopsis*	*35S*	Drought and freezing	40
OsDREB1A	AP2/ERF	Rice	*Arabidopsis*	*35S*	Drought, salt, and freezing	8
Tsi1	AP2/ERF	Tobacco	Tobacco	*35S*	Salt	38
JERF1	AP2/ERF	Tomato	Tobacco	*35S*	Salt	58
ICE1	HLH	*Arabidopsis*	*Arabidopsis*	Superpromoter*	Freezing	6
AtMYC2 & AtMYB2	MYC & MYB	*Arabidopsis*	*Arabidopsis*	*35S*	Osmotic stress	2
CpMYB10	MYB	*Craterostigma plantagineum*	*Arabidopsis*	*35S*	Drought and salt	55
Osmyb4	MYB	Rice	*Arabidopsis*	*35S*	Cold and freezing tolerance	54
AREB1/ABF2	bZIP	*Arabidopsis*	*Arabidopsis*	*35S*	Drought, salt, heat, and oxidative stress	22
AREB2/ABF4	bZIP	*Arabidopsis*	*Arabidopsis*	*35S*	Drought, salt, chilling, freezing, heat, and oxidative stress	18, 22
ABF3	bZIP	*Arabidopsis*	*Arabidopsis*	*35S*	Drought, salt, chilling, freezing, heat, and oxidative stress	18, 22
ABI5	bZIP	*Arabidopsis*	*Arabidopsis*	*35S*	Water stress	27
ABI3 (plus ABA)	VP1	*Arabidopsis*	*Arabidopsis*	*35S*	Freezing	51
SCOF-1	Zn finger	Soybean	*Arabidopsis*	*35S*	Low temperature stress	21
			Tobacco	*35S*	Low temperature stress	21
STZ	Zn finger	*Arabidopsis*	*Arabidopsis*	*35S*	Drought	41
ANAC019/ANAC	NAC	*Arabidopsis*	*Arabidopsis*	*35S*	Drought	52
ANAC055/AtNAC3	NAC	*Arabidopsis*	*Arabidopsis*	*35S*	Drought	52
ANAC072/RD26	NAC	*Arabidopsis*	*Arabidopsis*	*35S*	Drought	52

* Superpromoter consists of three copies of the octopine synthese upstream-activating sequence in front of the manopine synthese promoter.

Application of regulon biotechnology to improve stress tolerance in crop plants

The orthologous genes of *DREB1/CBF* have been found in many crop plants such as canola, broccoli, tomato, alfalfa, wheat, barley, corn, and rice[59]. These indicate that the DREB1/CBF regulon system is ubiquitous in the plant kingdom, and the "DREB technology" with controlling the expression of the DREB1/CBF regulon system is expected to improve the tolerance against stresses in crop plants. So far the *DREB1/CBF* genes of *Arabidopsis* have been successfully used to engineer abiotic stress tolerance in a number of different species (Table 1). For example, constitutive overexpression of the *Arabidopsis DREB1/CBF* genes in canola results in increased freezing tolerance[16] and drought tolerance[59].

We have isolated rice orthologs for DREB1/CBF and DREB2, four OsDREB1s and one OsDREB2, in the rice genome sequence and they function in stress-inducible gene expression[8]. Overexpression of OsDREB1A in *Arabidopsis* revealed that this gene has a similar function to that of its *Arabidopsis* homolog in stress-responsive gene expression and stress tolerance. This indicates that similar transcription factors function in dicotyledons and monocotyledons. A novel DREB1/CBF transcription factor ZmDREB1A was also identified in *Zea mays*[40]. The ZmDREB1A was involved in cold-responsive gene expression and overexpression of the *ZmDREB1A* gene in *Arabidopsis* which resulted in increased drought and freezing tolerance.

However, constitutive overexpression of the *DREB1/CBF* genes in plants showed dwarf phenotype[11,26]. To overcome these problems, stress-inducible promoters that have low background expression under normal growth condition have been used in conjunction with the *DREB1/CBF* genes to achieve increased stress tolerance without the growth retardation[19,25]. Constitutive overexpression of *Arabidopsis DREB1A/CBF3* improved drought- and low-temperature stress tolerance in tobacco[20]. The stress-inducible *RD29A* promoter minimized the negative effects on the plant growth in tobacco. Furthermore, we detected overexpression of stress-inducible target genes of DREB1A/CBF3 in tobacco. The *Arabidopsis DREB1A/CBF3* gene was placed under control of the *RD29A* promoter and transferred via biolistic transformation into bread wheat[39]. Plants expressing the *DREB1A/CBF3* gene demonstrated substantial resistance to water stress in comparison through checks under experimental greenhouse conditions, manifested by a 10-day delay in wilting when water was withheld. These results indicate that a combination of the *RD29A* promoter and *DREB1A* is useful for improvement of various kinds of transgenic plants that are tolerant to environmental stress.

Now we collaborate with many research groups to improve stress tolerant crop plants utilizing regulon biotechnology (Fig. 2). We hope the results of these collaborative studies will contribute to the sustainable food production in developing countries and help to prevent the global-scale environmental damage.

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Water Resources: Agriculture, the Environment, and Society

An assessment of the status of water resources

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Water is a renewable resource, but its availability is variable and limited. Nearly every country in the world experiences water shortages during certain times of the year (Gleick 1993a), and more than 80 countries now suffer from serious water shortages (Falkenmark and Lindh 1993).

Factors such as rainfall, temperature, evaporation, and runoff determine water availability. Clean water resources per capita are declining rapidly as the needs of the growing population increase (Pimentel et al. 1994). Population growth not only reduces water availability per person but stresses the entire environmental system. As the world population increased from 3.8 billion to 5.4 billion during the last two decades, water use worldwide increased threefold (Postel 1992). In addition, factors such as pollution, erosion, runoff, and salinization associated with irrigation, plus the overall inefficient use of water, contribute to the decline in water resources (Pimentel et al. 1994).

Major difficulties exist in allocating the world's scarce freshwater resources. These problems exist between and within countries, between industries, and between individual communities. Consider that agriculture alone consumes 87% of the fresh water withdrawn in the world (Postel 1992). Water shortages can be expected to severely reduce biodiversity in both aquatic and terrestrial ecosystems (Postel et al. 1996).

New water supplies likely will result from conservation, recycling, reuse, and improved water use efficiency rather than from large development projects

In this assessment of the status of water resources, we analyze consumption of water by individuals, agriculture, and energy production. We also address the relationship of water availability to biodiversity. Finally, considering projected population growth, climate change, and water use patterns, we suggest strategies for improving water use to meet the increasing and conflicting needs of agriculture, society, and the environment in future decades.

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Water resources

Water resources depend on the hydrologic cycle, on climate change, and to some degree on fossil water in the ground.

Hydrologic cycle. The earth's atmosphere contains approximately 1.3×10^{13} m^3 of water and is the source of all of the rain that falls on Earth. Each year, approximately 151,000 quads (1 quad = 10^{15} BTU) of solar energy distills and moves 5×10^{14} m^3 of water from the earth's surface into the atmosphere—86% from oceans and 14% from land (Postel 1985). This is approximately 400 times the total amount of fossil energy (320 quads) burned each year in the world. Although only 14% of the evaporation occurs from land, approximately 24% (1.2×10^{14} m^3 per year) of the world's precipitation falls onto land (Shiklomanov 1993). The sun's energy therefore transfers a significant portion of the water from the oceans to the continents each year.

The 1.3×10^{13} m^3 of water in the atmosphere is a small percentage (0.001%) of the 1.4×10^{18} m^3 water estimated to be in the oceans (WRI 1989). The total amount of fresh water held on the surface of the earth in streams and lakes is approximately 1×10^{14} m^3. This represents approximately 0.3% of the total fresh water on Earth, including water in ice caps, glaciers, ground water, and as soil moisture. Approximately 23% of the total fresh water on Earth is stored as ground water, which is 82 times more abundant than the amount of fresh water in streams and lakes (Shiklomanov 1993).

Groundwater resources. Ground water has accumulated over many

millions of years in aquifers located below the surface of the earth. An estimated 8.2×10^{15} m^3 of water are now present in the world's aquifers (WRI 1989). Most aquifers are replenished slowly, with an average recharge rate that ranges from 0.1% to 0.3% per year (Covich 1993). Assuming an average 0.2% recharge rate, only 16.4×10^{12} m^3 of water per year is available for sustainable use. The amount of fresh water stored in the top 0.2 m of world soil is estimated to be 16.5×10^{12} m^3 (Levine et al. 1979). On average, water accounts for nearly one-quarter of the weight of the upper-soil level.

In the United States, ground water provides approximately 46% of the water used by all households (Solley et al. 1993), although approximately 97% of the water used by rural households is from groundwater sources (NGWPF 1987). Irrigation for agriculture also relies heavily on ground water. For example, 66% of irrigation water in Texas and 38% in California is pumped from ground water (Solley et al. 1993).

Population growth and the associated increase in irrigated agriculture have led to the mining of groundwater resources—that is, the rate of water withdrawal is considerably faster than the recharge rate, causing water tables in the United States to fall approximately 3–120 cm/yr in some irrigated regions (Sloggett and Dickason 1986). The Ogallala aquifer, which underlies parts of Nebraska, Kansas, Colorado, Oklahoma, New Mexico, and Texas is nearly half depleted, and recharge rates are only a small fraction of the withdrawal rates (Soule and Piper 1992, Thomas 1987). In Beijing, China, water tables are falling at a rate of 1 to 2 m/yr (Postel 1992). The rapid depletion of ground water by irrigation poses serious threats to the sustainable use of groundwater supplies in rural and irrigated farming regions.

Water availability. Although water is a renewable resource, its availability is finite in terms of the amount available per unit time in various regions of the earth (Table 1). Overall water availability is affected by many factors, such as the amounts and patterns of rainfall, substrate qualities, temperature, evaporation rate, vegetation cover, and runoff. The average precipitation for most continents is approximately 700 mm/yr (7 million liters/ha). Australia and South America vary from this figure significantly, with 450 mm/yr and 1600 mm/yr, respectively (Shiklomanov 1993). Africa, however, is relatively arid despite having a similar average rainfall to other continents because its warm climate causes 80% of its water to evaporate before it is available for use (Budyko 1986).

Table 1. Regions of the world with water problems (based on the criterion that yearly water availability per capita is less than 1000 m^3/yr) and their per capita water availability (Falkenmark and Lindh 1993).

Region	Water availability per capita (m^3/yr)
Egypt	40
Malta	50
West Bank	126
Gaza Strip	133
Yemen	220
Jordan	255
Israel	376
Saudi Arabia	300
Libya	300
Hai-Luan River Basin, China	308
Huai He River Basin, China	424
Syria	440
Tunisia	600
Kenya	610

Some areas simply receive insufficient rainfall (less than 500 mm/yr) for agriculture, which leads to serious water and food problems. Of the 14 Middle Eastern countries, nine are facing shortages of water (less than 1000 m^3 per person per year; Postel 1992). Egypt, which receives practically no rainfall and also has a high rate of evaporation, depends almost totally (97%) on the lower Nile, which flows in from its upstream neighbors, including the Sudan and Ethiopia.

Some hydrologists define water scarcity based on the flow or runoff in rivers after evaporative losses (Falkenmark and Lindh 1993). In general, a nation is considered water scarce when the availability of water drops below 1000 m^3 per person per year (2740 liters per person per day; Table 1). Water stress occurs when water availability ranges from 1000 to 1700 m^3 per person per year (Engelman and LeRoy 1993). The result is a lack of water for irrigation, industry, and protection of the environment. Twenty-six nations, including Egypt, Jordan, Israel, Syria, Iraq, Iran, and Saudi Arabia, are currently defined as water scarce (Engelman and LeRoy 1993, Postel 1992). At least 11 countries experience water stress, including Ethiopia, Kenya, Somalia, Algeria, and Libya.

Even in countries that possess abundant water resources, such as the United States, many existing sources of water are being stressed by withdrawals from groundwater wells and diversions from rivers and reservoirs to meet the needs of homes, cities, farms, and industries.[1] Increasing requirements to leave water in streams and rivers to meet environmental, human, and recreational needs further complicate the problem. In the past, water management in the United States has focused on developing ways to exploit the country's supplies of fresh water. Many large dams were built during the early twentieth century to increase the supply of fresh water. The era of constructing large dams and conveyance systems to meet water demand in the United States is drawing to a close; the limited water supply and established infrastructure require that demand be managed effectively within the available supply (Figure 1). New water supplies likely will result from conservation, recycling, reuse, and improved water use efficiency rather than from large development projects, as in the past (Postel et al. 1996).

Climate and other human-induced environmental changes. Estimates of water resources and their availability are based on present world climate patterns. However, the continued loss of forests and other vegetation and the accumulation of carbon dioxide, methane gas, and nitrous oxides in the atmosphere may

[1] W. Solley, 1995, personal communication. US Department of the Interior, US Geological Survey, Washington, DC.

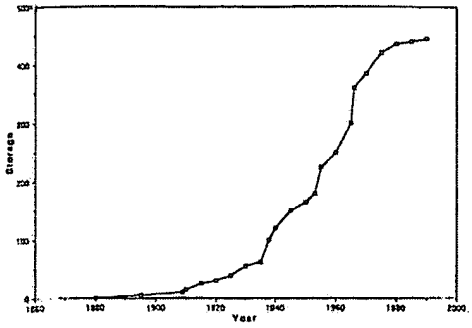

Figure 1. Total reservoir storage capacity (in millions of acre-feet; 1 acre-foot = 1231 m^3) in the United States from 1880 to 1990 (USGS 1995).

lead to global warming, which would change precipitation and temperature patterns (Downing and Parry 1994). Meeting agricultural and other societal needs for fresh water will become even more difficult in some regions; however, in other regions benefits might accrue because of warmer temperatures and higher rainfall for agriculture.

For example, California, which is already experiencing water shortages, is likely to have a 20%–40% decrease in mountain snowpack and water flow through its river basins (Vaux 1991). And the snowpack would most likely melt earlier in the year, creating early summer shortages and more severe water shortages late in the summer (Vaux 1991). On the other hand, areas like Canada may benefit from warming, which would create longer growing seasons; however, these areas may be faced with water shortages (Parry and Carter 1989).

If mean annual temperatures rise 3°–4°C, rainfall in the US corn belt region is projected to decline by approximately 10% (Downing and Parry 1994). Low rainfall and increased evaporation rates would combine to limit corn production in the region (Rosenzweig and Parry 1994). The predicted global warming could increase world irrigation needs by 26% to maintain current production (Postel 1989). In addition to global warming, population growth and associated activities may influence water resources through other environmental changes, such as deforestation, desertification, soil erosion, and loss of biodiversity (Heywood 1995).

Water use

Water resources are withdrawn for use and consumption in many human activities. The term *use* implies that some of the withdrawn water is returned for reuse, for example, cooking water or wash water. In contrast, *consumption* means that the water is nonrecoverable and is not returned to the water resource.

Human use of water. The water content of all living organisms ranges from 60% to 95%. Humans need to consume 1 to 2 liters of water per day per person (Watson 1988). Americans use approximately 400 liters of water per person per day for drinking, cooking, washing, disposing of wastes, and other personal uses (USBC 1994). This use is much higher than the average world personal use of 90 liters per person per day (Brewster 1987). Worldwide, total use of fresh water averages approximately 1800 liters per person per day for all uses (WRI 1991).

Total US freshwater withdrawals are approximately 1280 billion liters per day, or approximately 5100 liters per person per day including water use in irrigation, with 77% coming from surface water and 23% withdrawn from ground water (Solley et al. 1993). In China, only approximately 1100 liters per person per day are withdrawn for all purposes, one-fourth of the use in the United States (Zhang 1990). Postel et al. (1996) report that humankind now uses 26% of total evapotranspiration and 54% of all runoff in rivers, lakes, and other accessible sources.

Crop production. Agricultural production consumes more fresh water than any other human activity (Falkenmark 1989). US agriculture accounts for 87% of the fresh water consumed after being withdrawn (Figure 2). Plants render all water that passes through them nonrecoverable through evaporation and transpiration. In the United States, approximately 62% of the water used in agriculture comes from surface sources and 38% comes from groundwater sources (Solley et al. 1993). Approximately 68% of all ground water withdrawn in the

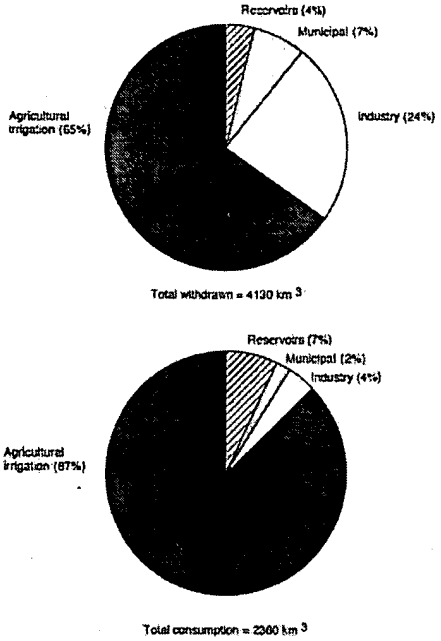

Figure 2. Annual human water use worldwide (data from Shiklomanov 1993). Water withdrawn refers to water pumped, and consumption refers to water that is used and is nonrecoverable (per year).

United States is used for agriculture (Solley et al. 1993).

Different crops and regions vary in their water requirements. Rainfall patterns, temperature, soil quality, and vegetative cover all influence soil moisture levels. For ideal growing conditions, soil moisture should not fall below 50% in the root zone (Blackshaw 1990), but for some crops, like rice, more than 50% is needed for full yields (Bhuiyan 1992). Good vegetative cover, high levels of soil organic matter, active soil biota, and slow water runoff increase the percolation of rainfall into the soil for use by growing crops.

The transfer of water to the atmosphere from the terrestrial environment by transpiration through vegetation is estimated to range between 38% and 65% of precipitation depending on the terrestrial ecosystem.[2] The processes of carbon dioxide fixation and temperature control require plants to transpire enormous amounts of water. For example, a squash plant transpires ten times its fresh weight in

[2]T. Dawson, 1995, personal communication. Cornell University, Ithaca, NY.

water per day, and many deciduous trees transpire two to six times their fresh weight per day.

The water required to grow various food and forage crops ranges from 500 to 2000 liters of water per kg of yield produced (Table 2). For instance, 1 ha of US corn transpires approximately 4 million liters (4000 m^3/ha) of water during its growing season, and an additional 2 million liters/ha evaporate concurrently from the soil (Donahue et al. 1990). Thus, during the growing season approximately 600 mm (6 million liters/ha) of rainfall is needed for corn production. Even with 800–1000 mm (8–10 million liters/ha) of annual rainfall in the corn belt region, corn usually suffers from lack of water at some point during the summer growing season (Troeh and Thompson 1993).

High-yielding rice requires much more water for production, from 10 to 18 million liters of water/ha. Up to 50% more rice per hectare is produced under flooded conditions than under sprinkler irrigation.

The biomass and total yields of soybeans (2.3×10^3 kg/ha) and wheat (2.7×10^3 kg/ha) produce less biomass and total yields than corn (7.6×10^3 kg/ha) or rice (6.2×10^3 kg/ha) on average (USDA 1993). Nevertheless, soybeans are highly consumptive of water, requiring approximately 4.6 million liters/ha for transpiration. Wheat, by contrast, requires only approximately 2.4 million liters/ha.

Sorghum and millet production require only 250–300 mm (2.5–3 million liters/ha) of annual rainfall (Gleick 1993a), and some cereal production can take place with annual rainfall levels as low as 200–250 mm (Rees et al. 1990). Under these relatively arid conditions, crop yields are low ($1–2.5 \times 10^3$ kg/ha), even with adequate amounts of fertilizer (USDA 1993).

US agricultural production is projected to expand because of increased food needs and the increase in population. The projected 30% increase in US crop and livestock production during the next two decades will significantly stress water resources in the central United States. Increasing crop yields carries a parallel increase in freshwater consumption in agriculture.

Table 2. Estimated liters of water required to produce 1 kg of food and forage crops.

Crop	Liters/kg
Potatoes	500
Wheat	900
Alfalfa	900
Sorghum	1110
Corn	1400
Rice	1912
Soybeans	2000
Broiler chicken	3500
Beef	100,000

Livestock production. Producing 1 kg of animal protein requires approximately 100 times more water than producing 1 kg of vegetable protein (Pimentel and Pimentel 1996). In the United States, livestock directly use only 1.3% of the total water used in agriculture, but including the water required for forage and grain production greatly increases the water requirements for livestock production. Producing 1 kg of beef requires approximately 100 kg of hay-forage and 4 kg of grain (Pimentel et al. 1980). Producing this much forage and grain requires approximately 100,000 liters of water to produce approximately 100 kg of plant biomass plus 5400 liters to produce 4 kg of grain (Falkenmark 1994). On rangeland, more than 200,000 liters of water are needed to produce 1 kg of beef (Thomas 1987). Forage, some cereals, and livestock can be produced with rainfalls of only 150 to 200 mm per year (1.5–2 million liters/ha; Rees et al. 1990, USDA 1993), but production is low under such arid conditions.

Animals vary in the amounts of water required for their production. In contrast to beef, 1 kg of broiler chickens can be produced with approximately 2.5 kg of grain requiring approximately 3500 liters of water.

Irrigation and energy use. Both water and energy resources are expended in the irrigation of arid land to make them productive. Approximately 16% of the world's cropland is irrigated (WRI 1992) and approximately 33% of the world's food is produced on this irrigated land (Postel 1992). Worldwide, the amount of land under irrigation is slowly expanding, even though salinization, waterlogging, and siltation are decreasing productivity of some irrigated lands.[3] Despite a small annual increase in irrigated areas, the per capita irrigated area has been declining since 1978 (Postel 1992). For example, per capita irrigated land in the United States has declined 8% between 1978 and 1988 (USDA 1993).

Irrigation requires a significant amount of energy for pumping and moving irrigation water. Annually, approximately 120×10^{12} kcal/yr, or approximately 10% of the total energy expended each year in US crop and livestock production, is used to pump water for irrigation. Partially irrigated wheat uses 4.2 times more energy than rainfed wheat, and in the US, irrigated alfalfa requires three times more energy than if it is rainfed (Singh and Mittal 1992). In Nebraska, irrigated corn production requires more than three times the energy of rainfed corn production (Pimentel 1980). Delivering the 7 million liters of irrigation water needed by a hectare of irrigated corn from surface water sources requires approximately 8 million kcal of fossil fuel (Pimentel 1980). This irrigation energy is 1.5 times the total of all other energy inputs required for corn production. If the water has to be pumped from a depth of 100 m, the energy cost rises to approximately 24,500 kcal, more than three times the energy cost of surface water (Gleick 1993a). In some areas, water must be pumped from 200 m, requiring a much greater fossil energy investment. Furthermore, the dollar cost of delivering 7 million liters of water per hectare from a depth of 30 m is approximately $1000/ha (Hinz 1985). Few crops are sufficiently valuable to justify spending $1000/ha for irrigation alone. In regions with falling groundwater tables, the high costs of pumping ground water eventually make crop production progressively less economical.

In total, approximately 10 million kcal are expended for machinery, fuel, fertilizers, pesticides, par-

[3]D. Haith, 1994, personal communication. Cornell University, Ithaca, NY.

'tial irrigation, and other inputs to produce 1 ha of corn in the United States (Pimentel and Wen 1990). The large quantities of energy needed to pump irrigation water place significant demands on energy and water resource management. This factor can be expected to influence the economics of irrigated crops and selection of specific crops worth irrigating.

Water use in energy production. Producing energy for all types of fuel requires pumping large quantities of water. Based on the current per capita use of electricity (24,300 kWh; USBC 1994) in the United States, the amount of water consumed by a conventional coal-generating plant is 29 m^3 per capita per year (Gleick 1994a). If cooling towers are used, water consumption jumps to 63 m^3 per capita per year. For an average hydroelectric plant, water consumption increases to 413 m^3 per capita per year, largely due to evaporation from the reservoirs (Gleick 1994a). To produce a total of 1 million kWh requires 1190 m^3, 2590 m^3, and 16,930 m^3 (Gleick 1994a), respectively, for a conventional plant, a plant with cooling towers, and a hydroelectric plant.

Electric power production requires large amounts of water to be pumped. For example, thermoelectric power generation withdraws approximately the same amount of US water as agriculture (496 million m^3/day versus 534 million m^3/day; Solley et al. 1993) but presents no significant consumption problems because 97% of the water that is withdrawn is returned directly to its source (Solley et al. 1993).

Currently, for example, with onshore oil extraction, between 0.2 m^3 and 1.2 m^3 of water must be withdrawn for the amount of oil (37,800 kWh, or 32.5 million kcal) that is consumed per capita per year (Table 3; USBC 1994). When the world's sources of oil and natural gas are depleted, other sources of fuel may have to be developed. Oil shale, coal gasification, or coal liquification will require pumping between 20 and 50 times more water to produce an equivalent amount of energy as surface mining (Table 3). For instance, oil produced from oil shale by mine retort requires 110–220 m^3 of water to produce 1 million kWh (Table 3). Producing the same amount of oil by enhanced oil recovery requires pumping 433 m^3 water per million kWh produced.

Table 3. Withdrawal of water for energy production and electricity generation (after Gleick 1994a).

Energy technology	Water used for energy production[a] ($m^3/10^6$ kWh)	Water used for electricity generation ($m^3/10^6$ kWh)
Nuclear		
Open pit mining	72	NA[b]
Uranium milling	29–36	NA
Nuclear fuel reprocessing	180	NA
Nuclear plant Light Water Reactor	NA	3200
Coal		
Surface mining and revegetation	7	NA
Other plant operations	325	NA
Coal combustion (cooling towers)	NA	2600
Coal gasification	140–340	NA
Coal liquification	120–250	NA
Oil		
Onshore oil extraction and production	6–29	NA
Enhanced oil recovery	433	NA
Oil combustion (cooling towers)	NA	2600
Oil shale (mine retort)	110–220	NA
Hydroelectric	NA	17,000

[a]Nuclear and fossil fuel production.
[b]Not applicable.

Conflicts over water resources. The rapid rise in consumption of fresh water for food production and other uses has spurred conflicts over water resources. At least 20 nations obtain more than half of their water from rivers that cross national boundaries (Gleick 1993a), and 14 countries receive 70% or more of their surface water resources from rivers that are totally outside their borders. For example, Egypt obtains 97% of its fresh water from the River Nile, which originates in other countries, including the Sudan; for Botswana, the percentage of water obtained from rivers outside its borders is 94%; and for Syria it is 79% (Gleick 1993a). Approximately 47% of the surface water located in international drainage basins is shared by two or more countries (McCaffrey 1993) and this water supports approximately 40% of the world's population (Postel 1993). In addition, many underground aquifers are shared by several countries.

Historically, countries in the Middle East have had the most conflicts over water, largely because they have less water per capita than other world regions (Gleick 1994b, c) and because every major river in the region crosses international borders (Gleick 1993b). Furthermore, their populations are increasing rapidly, some having doubled in the last 20 to 25 years (PRB 1995). The combination of water scarcity and population growth ensures that conflicts, like Syria's diversion of the Jordan headwaters, which played a leading role in the June 1967 Arab and Israeli War (Gleick 1993a), will continue.

Many conflicts have erupted on the African continent over the use of the Nile River (Gleick 1994c). This river, the second longest in the world, is shared by the Sudan, Ethiopia, Egypt, Burundi, Kenya, Rwanda, Tanzania, Zaire, Eritrea, and Uganda. The Nile is so dammed and overused that for parts of the year little or no fresh water reaches the sea (Postel 1995). The Egyptians have been using the Nile for irrigation for 5500 years, but until recently other nations in the upper drainage basin have made little use of Nile water (McCaffrey 1993). However, all the nations through which the Nile passes are becoming increasingly dependent on it as their populations increase and their food situations worsen.

Distribution of river water has also created conflicts between several states of the United States, Mexico, and Canada (Gleick 1994c). For instance, the Colorado River is shared by several states, including California, Nevada, Colorado, New Mexico, Utah, and Arizona. Because these states depend heavily on the river water, the Colorado River is a trickle by the time it reaches the Gulf of California and after Mexico takes its share of the water (Postel 1995).

Dam construction on the Ganges River has also led to water rights disputes. The Ganges River arises in the Himalayas and flows through India and eventually into Bangladesh. Between 1961 and 1975, India constructed a dam to divert water to a tributary that carries water to Calcutta (McCaffrey 1993). This dam has reduced the flow of water to Bangladesh, where it is needed for irrigation. In addition, the natural flow of the Ganges is essential to prevent siltation and flooding in Bangladesh and to prevent salt water intrusion from the Bay of Bengal. India's continued diversion of more and more water from the Ganges has recently led to riots and protests in Bangladesh (Khurshida 1989).

Limits to water use

Utilization of fresh water is limited by pollution, economic costs, and land management practices.

Costs of water treatment. Surface and groundwater pollution not only pose a threat to public and environmental health, but the high cost of water treatments further limits the availability of water for use. Depending on the water quality and the purification treatment used, potable water costs from 30¢ to 33¢ per 1000 liters (Table 4; Gleick 1993a).[4] If the water has to be run through charcoal filters for further purification, it costs an additional 13¢ per 1000 liters. If salts also have to be removed from the water, costs rise by an additional 21¢ per 1000 liters (EPA 1980). In the United States, the cost of treating sewage for release into streams and lakes ranges from 8¢ to 10¢ per 1000 liters (EPA 1980, Gleick 1993a). If salt water from oceans has to be desalinated, the costs are enormous, ranging up to $2 per 1000 liters (Table 4). At this rate, for an average per capita use of water of 5100 liters/day (the total US freshwater withdrawal rate), it would cost approximately $10 per person per day to use desalinated water. In some cases, sewage effluent might be used for crop and forage production, and this would reduce overall water costs (Tanji and Enos 1994).

Table 4. Cost of delivering 1000 liters of clean, safe water in the United States.

Treatment	Cost	Source
Chlorination	0.3–17¢	Troyan and Hanson 1989
Sewage treatment	8–10¢	EPA 1980, Gleick 1993a
Activated carbon	13¢	Gammie and Giesbreck 1986
Removing salts	21¢	EPA 1980
Potable water (processing)	30–33¢	Unpublished data[a]
Desalinization (ocean water)	Approximately $2.00	Gruen 1994, Ingram 1991, Kally 1994

[a]J. Rogers, 1980, personal communication. Village of Cayuga Heights, NY.

Cleaning water and reducing the BOD (biological oxygen demand) is energy costly; removing 1 kg of BOD requires 1 kWh (Trobish 1992). Most of the cost of cleaning water arises from the energy and equipment costs involved in pumping and delivering water. Delivering 1 m^3 (1000 liters or 1 metric ton) of water in the United States requires the expenditure of approximately 1140 kcal. Excluding the energy for pumping sewage, the amount of energy required to process 1 m^3 in a tertiary waste water treatment plant requires approximately 70 kcal of energy.[5] The cost of water treatment and the energy used to purify water will most likely increase in the future as population growth increases both water pollution and water demand.

Economic costs and subsidies for water. Because the high cost of treating and delivering water can limit its availability and use, many governments throughout the world subsidize water for agriculture. For example, farmers in California pay as little as 0.5¢ per m^3, whereas the public pays approximately 25¢ per m^3 (Bolling 1990). If US farmers paid the full cost of water, they would probably manage irrigation water more effectively (LaVeen and King 1985).

The 12% of US cropland that is irrigated produces 27% of the dollar value of all crops (USDA 1993). However, this 27% dollar value does not take into account the costs to the government of supplying and subsidizing a large portion of US irrigation water. The construction cost subsidy for western irrigation is approximately $487O/ha, which represents an annual construction cost subsidy of approximately $440 · ha^{-1} · yr^{-1} over the life of the project (US Congress 1989). An annual $540/ha is added to the construction cost for water and power for moving water (LaVeen and King 1985). Thus, the total subsidy for irrigation per hectare in the western region of the United States is approximately $980 · ha^{-1} · yr^{-1}; based on these data, this irrigation costs approximately 10¢/m^3. The total annual government subsidy is estimated to be approximately $4.4 billion for the 4.5 million ha irrigated in the West.

Other nations have similar patterns of subsidies. For example, farmers in Mexico pay only 11% of their water's full cost, and farmers in Indonesia and Pakistan pay only 13% (Postel 1992). Such undercharging for irrigation water in the United States and other nations encourages the planting of relatively low-value crops and the inefficient use of water. In general, vegetable and fruit crops return more per dollar invested in irrigation water than field crops. For example, in Israel 1 m^3 of water from irrigation produces 79¢ worth of groundnuts and 57¢ worth of tomatoes but only 13¢ worth of corn grain and 12¢ worth of wheat (Fishelson 1994).

[4]J. Rogers, 1980, personal communication. Village of Cayuga Heights, NY.

[5]B. Cross, 1994, personal communication. Village of Cayuga Heights, NY.

As fossil energy prices increase, irrigation costs also increase. For example, from 1973 to 1983 US irrigation costs increased from $551 million to $2.5 billion per year (Sloggett 1985). As a result, many US farmers who had been irrigating low-value crops such as alfalfa switched to high-value crops such as cotton, lettuce, and strawberries (Lacewell and Collins 1986).

Improving agricultural water use. Coupled with inefficient crop choice, some irrigation practices waste large amounts of water. Most farmers use flooding or channeling methods to irrigate their crops; thus, irrigation efficiency, or the amount of water reaching the crop, worldwide is estimated to be less than 40%. Large amounts of water are lost through pumping and transporting (Postel 1992). In the United States, less than 50% of irrigation water actually reaches the crop (van der Leeden et al. 1990).

Although improving irrigation efficiency is difficult, conservation technologies can improve irrigation and reduce the irrigation water needed to produce the same crop yield. For example, some farmers are turning to "surge flow" irrigation to replace traditional flooding and channeling irrigation (Verplaneke et al. 1992). This practice involves an automated gated-pipe irrigation system that uses a microprocessor control instead of releasing water in a continuous, slow stream in field channels. Using this method, farmers in Texas have been able to reduce water pumping 38% to 56%, compared with continuous flood irrigation of the same area (Sweeten and Jordan 1987).

Another strategy is irrigating at night to reduce evaporation. This technique improves irrigation efficiency by two to three times (Dubenok and Nesvat 1992). The use of low-pressure sprinklers also may improve water efficiency by 60% to 70%, compared with high-pressure sprinklers (Verplaneke et al. 1992). Avoiding overhead watering can reduce evaporation and water needs by 45% (O'Keefe 1992).

Low-Energy Precision Application (LEPA) is another technique for conserving water. LEPA sprinklers deliver water to the crop by drop tubes that extend down to the crop from a sprinkler arm (Sweeten and Jordan 1987). Water application efficiency of the LEPA system ranges from 88% to 99% (Sweeten and Jordan 1987). Combined ridge-tillage (planting crops on top of permanent ridges) and LEPA can significantly increase irrigation efficiency (Lal 1994).

The "drip" or "microirrigation" technique developed in the 1960s has spread rapidly worldwide, especially to Israel, Australia, New Zealand, and some regions in the United States. Drip irrigation delivers water to each individual plant by plastic tubes. This method uses from 30% to 50% less water than surface irrigation (Tuijl 1993). Although drip systems achieve up to 95% efficiency, they are expensive and energy intensive, and relatively clean water is needed to prevent the clogging of the fine delivery tubes (Snyder 1989). A comparison of drip irrigation with subirrigation and seepage for tomato production in Florida indicated that drip irrigation reduced water needs by 50% but added $328/ha to the production costs (Pitts and Clark 1991).

Planting trees to serve as shelter belts reduces evaporation and transpiration from the crop ecosystem from 13% to 20% during the growing season (Mari et al. 1985). The resulting increase in crop yields ranges from 10% to 74% for corn (Gregersen et al. 1989). Furthermore, this practice can reduce wind erosion by as much as 50% (Troeh et al. 1991). Also, intercropping crops with trees, if they are "hydraulic lifters" (e.g., *Acer* and *Eucalyptus*), may increase water availability for the crop as well as productivity.[6]

Runoff and erosion. Because crops require large quantities of water for their growth, it is vital that as much water as possible percolate into the soil instead of running off. Soil erosion often limits the amount of water available for crop use (Lal and Stewart 1990). When raindrops hit exposed soil they have an explosive effect, launching soil particles into the air. If the water does not percolate into the soil, it runs off and carries soil with it. More than half of the soil contained in the splashes is carried downhill on land with a slope greater than 1% (Foster et al. 1985). In most fields, raindrop splash and sheet erosion are the dominant forms of erosion (Foster et al. 1985). Eroded soils absorb from 10 to 300 mm $\cdot$ ha^{-1} $\cdot$ yr^{-1} less water, or from 7% to 44% of total rainfall (Pimentel et al. 1995). As expected, loss of rainwater severely reduces crop productivity. A runoff of even 20% to 30% of total rainfall can result in significant water shortages for crops and ultimately low crop yields (Elwell 1985).

Because soil erosion decreases both soil depth and its organic matter content, the ability of the remaining soil to retain water is significantly decreased (Fullen 1992). Thus, soil erosion is a self-degrading process—as erosion removes topsoil and organic matter, runoff intensifies and erosion worsens, only to be repeated with more intensity during subsequent rains. For example, Lal (1976) reported that in the tropics, erosion may reduce water infiltration by up to 93% and dramatically increase water runoff and loss. Water runoff and the transport of sediments, nutrients, and pesticides from agriculture to surface and ground waters are the leading cause of nonpoint source pollution in the United States (EPA 1994).

Water runoff and soil loss can be reduced by using vegetative cover, such as intercropping and ground cover (Lal 1993). For example, when silage corn is interplanted with red clover, water runoff can be reduced from 45% to 87% and soil loss can be reduced 46% to 78%, compared with silage corn grown without clover (Wall et al. 1991). Reducing water runoff in this way is an important step in increasing water availability to crops, conserving water, decreasing nonpoint pollution, and ultimately decreasing water shortages (NGS 1995).

Salinization and waterlogging. Dissolved salts in surface and ground water used for irrigation can increase soil salinity. Irrigation water that is applied to crops returns to the atmosphere via plant transpiration and evaporation, leaving the dissolved salts behind in the soil. Soil type, drainage, and water table depth

[6]See footnote 2.

all influence salt accumulation (Dinar and Zilberman 1991). Worldwide, approximately half of all irrigation systems are adversely affected by salinization (Szabolcs 1989). Agricultural land lost by salinization is estimated to be approximately 2 million ha/yr (Umali 1993).

Subsurface runoff and leaching from saline soil can increase salt levels in river and stream water. Some ground water and aquifers also accumulate salts after extensive irrigation (Stolte et al. 1992). As the Colorado River flows through the Grand River Valley in Colorado and water is withdrawn for irrigation, some is later returned to the river along with an estimated 18×10^3 kg/ha of salts leached from the irrigated land (EPA 1976). At times during the summer, the Red River in Texas and Oklahoma is more saline than seawater, because of leached salts (USWRC 1979). Disposal of saline water also has a detrimental impact on agriculture and aquatic species (Kelman and Qualset 1991). The addition of salts to water not only severely limits water use, but the process of desalinization is expensive.

The severity of water use and salinization is illustrated by the Aral Sea in Kazakhstan and Uzbekistan. The surface area of the Aral Sea has been reduced by nearly 50% and its volume by 75% during the last 33 years (Postel 1995), while its salinity has more than tripled (WRI 1994). The reduction and salinization in the Aral Sea was brought about by diversion of river water for irrigation primarily for cotton production (WRI 1994).

Waterlogging is another problem associated with irrigation. Over time, seepage from canals and irrigation of fields results in the rise of water tables and waterlogging. In the absence of adequate drainage, this water rises into the root zone and damages the growing crops. These waterlogged fields are sometimes referred to as "wet deserts" because they are rendered unproductive (Postel 1992). To prevent salinization and waterlogging, sufficient water and adequate drainage must be available to leach out salts and drain the excess water from the soil.

Conclusions

The availability and quality of fresh water has become a major international problem. Limited water resources and inefficient water use, combined with the rapidly growing world population, will further stress the world's finite freshwater supply. Competition for water within regions and countries continues to grow.

Water resources, along with fertile soil, energy, and biodiversity, are vital to maintaining the world's food supply. Agricultural production currently accounts for approximately 87% of the world's freshwater consumption. In the future, the need for and the use of water in agriculture will increase as the production of food and fiber is augmented to meet the needs of the expanding world population. In many parts of the world, per capita freshwater resources available for food production and for other human needs are declining and are becoming scarce in the arid regions. In the future, in arid regions where groundwater resources are the primary source of water, irrigation probably will have to be curtailed and the types of crops and livestock altered to meet the changing water situation.

To encourage conservation and to increase overall efficiency, subsidies for irrigation water should be phased out. Irrigation technologies that make efficient use of water for crop production must be encouraged. In general, more efficient use of water in agricultural production could be achieved by providing farmers with incentives to conserve water and soil resources. Controlling erosion also helps conserve water by reducing rapid water runoff. Protecting forests and other biological resources facilitates effective use of water resources and helps maintain the hydrologic cycle.

Most human activities adversely affect the quality of freshwater resources. Chemical and pathogen pollution of water supplies not only diminish the quality of water but cause human health problems. Agricultural and industrial chemicals and the lack of community sanitary facilities are the primary causes of water pollution. According to the World Health Organization (1992), approximately 90% of all illnesses in developing countries result from waterborne parasites and pathogens.

As fossil energy supplies decline, large amounts of water will be needed to make use of some less common forms of fossil energy, such as shale oil. New renewable energy technologies that require less water than existing fossil energy technologies need to be developed.

Water limits exist in many regions of the world even without the effects of the projected global warming. By causing changes in rainfall patterns and more rapid evaporation, global warming is likely to intensify the water crisis in many regions of the earth. Increased water stress, with or without global warming, is projected to have a negative impact on agricultural and forest production and other plant and animal species throughout the world ecosystem.

To avoid further water problems and lessen projected harsh outcomes for the future, humankind must conserve water and energy, and must protect land and biological resources—all of which are vital for a sustainable economy and environment. Humans can manage water resources more efficiently in agriculture and in other activities. Conservation of water and pollution control by individuals as well as by society is essential if the integrity of the water supply is to be protected.

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SHORT COMMUNICATION

The *Arabidopsis* homeobox gene *ATHB-7* is induced by water deficit and by abscisic acid

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Summary

Homeodomain-leucine zipper (HD-Zip) proteins are putative transcription factors encoded by a class of recently discovered homeobox genes as yet found only in plants. This paper reports on the characterization of one of these genes, *ATHB-7*, in *Arabidopsis thaliana*. *ATHB-7* transcripts were present in all organs of the plant at low levels, but expression was induced several-fold by water deficit, osmotic stress as well as by exogenous treatment with abscisic acid (ABA), a response being detectable at 10^{-8} M and reaching a maximum at 10^{-6} M ABA. The *ATHB-7* transcript was detected within 30 min after treatment with ABA and the transcript level was rapidly reduced after removal of the hormone. The induction of *ATHB-7* was shown to be mediated strictly via ABA, since no induction of *ATHB-7* was detectable in the ABA-deficient mutant *aba-3* subjected to drought treatment. Induction levels in two ABA-insensitive mutants *abi2* and *abi3* were similar to the wild-type response. In the *abi1* mutant, however, induction was impaired as 100-fold higher concentrations of ABA were required for a maximum induction as compared with wild-type. In this mutant the *ATHB-7* response was reduced also after drought and osmotic stress treatments. These results indicate that *ATHB-7* is transcriptionally regulated in an ABA-dependent manner and may act in a signal transduction pathway which mediates a drought response and also includes *ABI1*.

Introduction

Water availability is a major determinant of plant growth and development. In nature as well as in culture plants are exposed to highly variable water conditions. The plant response to variations in water supply includes a range of biochemical, physiological and morphological adaptations. Water deficit induces a rapid physiological response; stomata are closed in the leaves to prevent further water loss. Over a somewhat longer time perspective growth characteristics are adjusted; the leaf area is reduced and root growth increased.

The signal transduction pathways mediating these responses are poorly known. The plant hormone abscisic acid (ABA) appears to play a central role in the process (Mansfield, 1988). This conclusion was initially drawn from the observation of a stress-induced increase in endogenous ABA levels (Cohen and Bray, 1990) and from experiments using exogenous application of ABA which showed effects on plant growth and development resembling the environmental stress responses (for reviews see Mansfield, 1988; Trewavas and Jones, 1991; Zeevaart and Creelman, 1988). Under conditions of water deficiency, ABA is responsible for the onset of stomatal closure. This has been demonstrated by electrophysiological studies as well as by the inability of ABA-deficient *Arabidopsis* mutants to respond to water deficiency by stomatal closure (Koornneef *et al.*, 1982; Neill and Horgan, 1985).

ABA-deficient mutants (*aba*) in *Arabidopsis* were isolated in a screen for reversion of gibberellic acid-deficient (*ga*) mutant phenotypes (Koornneef *et al.*, 1982). Three alleles of the *ABA* locus exist (*aba-1*, *aba-3* and *aba-4*), and they are characterized by reduced seed dormancy, reduced stem length and leaf size, increased transpiration, wilting and a lowered ABA content. Mutation analysis has also identified a set of potential components of the transduction systems mediating the ABA response. Three classes of ABA-insensitive mutations, *abi1*, *abi2* and *abi3*, are defined by their ability to germinate in the presence of exogenous ABA at concentrations that inhibit germination of wild-type seeds (Koornneef *et al.*, 1984). Phenotypically, these mutants resemble the *aba* mutants in that they exhibit reduced seed dormancy. They all, however, have at least wild-type levels of endogenous ABA, indicating that their insensitivity to ABA does not result from an increased turnover or inactivation of the hormone, but from an impaired hormone sensitivity. The effects of the *abi3* mutation are essentially seed specific whereas the *abi1* and *abi2* mutations primarily affect vegetative growth. The *abi1* and *abi2* mutants have impaired stomatal regulation and are defective to varying extents in many ABA- and/or stress-regulated responses (Finkelstein and Somerville, 1990; Gilmour and Thomashow, 1991; Gosti *et al.*, 1995; Koornneef *et al.*, 1984; Nordin *et al.*, 1991; Schnall and Quatrano, 1992). Recently, two new ABA-insentitive mutants, *abi4* and *abi5*, have been identified (Finkelstein, 1994). They both show seed development-related phenotypes similar to the *abi3*

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mutant. Genetic analysis using double mutants of pairwise combinations of the *abi1*, *abi2* and *abi3* mutations (Finkelstein and Somerville, 1990) have suggested the existence of at least two partially overlapping ABA-related signal transduction pathways, one defined by *ABI3* and including also the *ABI4* and *ABI5* genes (Finkelstein, 1994), and a second pathway including the *ABI1* and *ABI2* genes.

A large number of genes which are regulated both by water deficit stress and/or exogenously applied ABA have been identified in different plant species (for reviews see Bray, 1993; Chandler and Robertson, 1994; Skriver and Mundy, 1990). These include the *LEA* genes, expressed also during the normal embryonic programme in association with seed desiccation (Dure *et al.*, 1981). The *LEA* genes, like a majority of genes induced by water stress are ABA responsive, but genes also exist which are inducible by wilting but not by ABA (Koizumi *et al.*, 1993). Recently, several lines of evidence demonstrate the existence of ABA-independent pathways mediating gene regulation in response to stress. For example, among genes that were differentially expressed in relation to water stress in *Arabidopsis*, Gosti *et al.* (1995), identified both ABA-responsive and ABA-non-responsive genes. How these pathways interact is not clear. The search for possible transcription factors that potentially mediate the ABA effect on gene expression is therefore of great interest.

In this paper, we report on the identification of a putative transcription factor which potentially is part of a transduction pathway mediating a drought stress response in *Arabidopsis*; the product of the homeobox gene *ATHB-7* (Söderman *et al.*, 1994).

ATHB-7 is a member of a recently discovered class of homeodomain-containing transcription factors, the HD-Zip proteins, identified in *Arabidopsis* (Mattsson *et al.*, 1992; Ruberti *et al.*, 1991; Schena and Davis, 1992), but present also in other plants (Chan and Gonzales, 1994; Kawahara *et al.*, 1995; Meissner and Theres, 1995). The number of different HD-Zip proteins in *Arabidopsis* is likely to exceed 15 (Söderman *et al.*, 1994). Unlike other homeodomain proteins identified, the HD-Zip proteins contain a leucine zipper in a conserved position carboxy-terminal to the DNA-binding homeodomain. Leucine zippers are known to mediate dimerization in the bZip class of transcription factors, including the oncoproteins Fos and Jun (Bush and Sassone-Corsi, 1990). Two *Arabidopsis* HD-Zip proteins, the *ATHB-1* and *ATHB-2* gene products, have been shown to bind DNA in a sequence-specific manner as homodimers (Sessa *et al.*, 1993).

The functions of the genes of the HD-Zip class are as yet largely unknown. Ectopic expression of the HD-Zip gene *HAT4* (which is closely related or identical to *ATHB-2*, Ruberti *et al.*, 1991) causes a series of developmental alterations in transgenic plants including increased hypocotyl elongation, early flowering, altered leaf morphology and dark green pigmentation (Schena *et al.*, 1993), suggesting an involvement of the gene in the phytochrome-mediated light control of development in *Arabidopsis*. This is consistent with the strong light dependence of expression recorded for *ATHB-2* (Carabelli *et al.*, 1993). Recent data on the expression of *ATHB-8* (Baima *et al.*, 1995) indicate this gene to function in the control of early stages of vascular tissue development.

We have previously reported on the expression patterns of four HD-Zip-encoding genes in *Arabidopsis*, *ATHB-3*, *ATHB-5*, *ATHB-6* and *ATHB-7* (Söderman *et al.*, 1994). All four genes were found to be expressed at low levels in the vegetative organs of the plant. In this report we present a detailed analysis of the expression of *ATHB-7*, which shows the gene to be regulated by water stress, by a mechanism which is dependent on abscisic acid as well as on *ABI1*. This suggests that *ATHB-7* may be part of a mechanism that controls the plant response to water deficit.

Results

Expression of ATHB-7 *is induced by water deficit, osmotic stress and treatment with exogenous abscisic acid*

We have previously reported that the *ATHB-7* gene is expressed at low levels in all organs of the plant, transcript levels being relatively high in leaves and flowers, and lower in roots, stems and seed pods (Söderman *et al.*, 1994). The low transcript level in the extracts of whole plants is confirmed by the Northern blot data shown in Figure 1 (lane C). This figure also shows the abundance of the *ATHB-7* transcript to be strongly dependent on the growth conditions of the plant; transcript levels being increased in seedlings exposed for 12 or 72 h to drought or to high-salt conditions, whereas low-temperature conditions had no effect. Even higher levels of transcript were observed in seedlings exposed to exogenous abscisic acid (ABA, Figure 1). In experiments using liquid root cultures of *Arabidopsis thaliana* a strong induction of *ATHB-7* was observed after treatment with exogenous ABA, but not after treatment with other hormones; gibberellic acid (GA3), the auxin indole acetic acid (IAA), or the cytokinin kinetin or by a combination of auxin and cytokinin (Figure 2). The effect of ABA was not affected by the simultaneous treatment with equimolar amounts of GA3, but a several-fold reduction in the induction level was observed at a 10-fold molar excess of GA3 over ABA (data not shown).

As shown in Figure 3(a), the *ATHB-7* response to ABA was detectable in seedlings within 30 min and reached a maximum at 2 h after addition followed by a progressive decrease from 4 to 12 h. Removal of ABA after a 2 h incubation resulted in a reduction in *ATHB-7* transcript levels, with a half life of approximately 30 min, the unin-

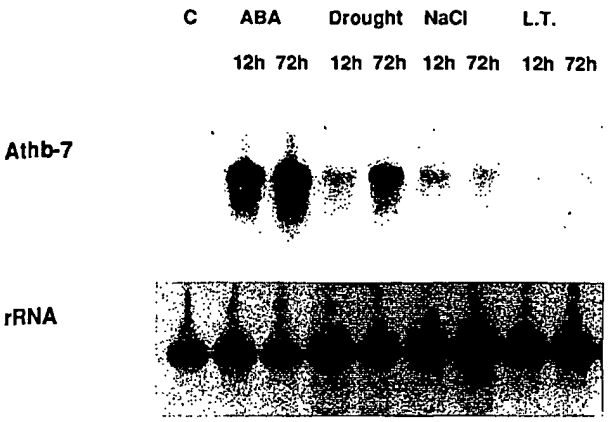

Figure 1. *ATHB-7* expression in seedlings exposed to drought, osmotic stress or to the plant hormone abscisic acid (ABA).
Northern blot analysis of total RNA, 30 μg per sample, from 10-day-old *Arabidopsis* seedlings exposed to ABA (10 μM), drought, NaCl (0.1 M) or low-temperature (LT) treatment for 12 or 72 h. C represents uninduced control seedlings. The filter was hybridized to a 516 bp *Hin*dIII–*Acc*I fragment of the *ATHB-7* clone. The lower panel shows a control hybridization of the same filter to an rRNA probe.

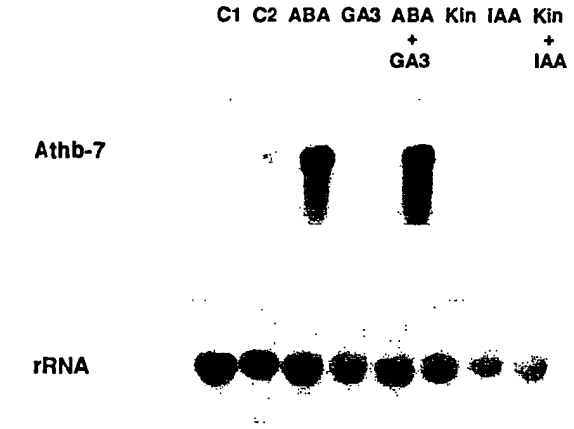

Figure 2. *ATHB-7* expression in response to plant hormones.
Northern blot analysis of total RNA, 12 μg per sample, from 14-day-old seedlings from liquid *Arabidopsis* cultures exposed to 10 μM abscisic acid (ABA), 10 μM giberillic acid (GA), 10 μM kinetin (Kin), 10 μM indole-3-acetic acid (IAA) or equimolar combinations of ABA+GA and kinetin+IAA. C1 is a water-treated control sample and C2 a sample derived from ethanol-treated control plants. The filter was hybridized to a 516 bp *Hin*dIII–*Acc*I fragment of the *ATHB-7* clone. The lower panel shows a control hybridization of the same filter to an rRNA probe.

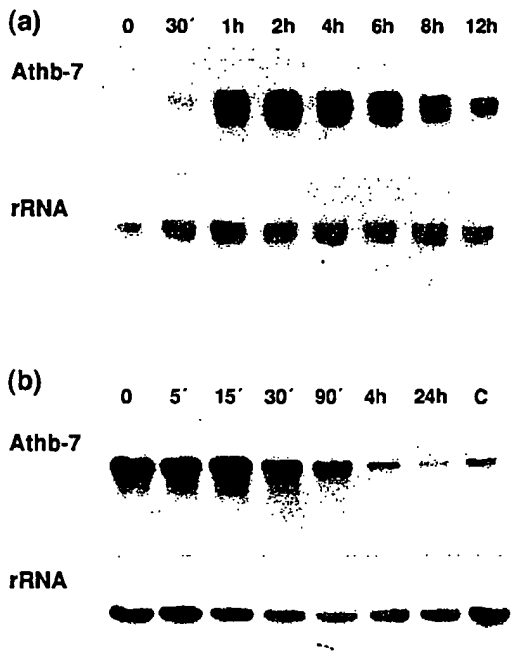

Figure 3. Time-course of the response of *ATHB-7* to ABA.
Northern blot analysis of 10 μg total RNA samples isolated from *Arabidopsis* seedlings from liquid cultures grown for 14 days and thereafter exposed to ABA. In (a), ABA (1 μM) was added to the medium and samples collected at different times after addition as indicated in the figure. In (b), ABA (1 μM) was added to the culture medium and the cultures incubated for 2 h at which time the medium was exchanged for medium without ABA. Samples were collected at different times after ABA removal as indicated in the figure. C indicates the untreated control. The filters were hybridized to a 516 bp *Hin*dIII–*Acc*I fragment of the *ATHB-7* clone. Lower panels in both (a) and (b) show control hybridizations of the filters to an rRNA probe.

duced background level being reached after 4 h (Figure 3b). The degree of induction was dependent on the concentration of ABA in the medium. An increase in *ATHB-7* transcript was recorded at a minimal concentration of approximately 10^{-8} M of ABA and a maximum response at approximately 10^{-6} M ABA (Figure 4a).

Induction of ATHB-7 *is reduced in the* abi1 *mutant.*

Koornneef and co-workers have isolated two classes of ABA mutants in *A. thaliana*; ABA-deficient (*aba*) mutants (Koornneef *et al.*, 1982) in which endogenous ABA levels are abnormally low in both seeds and whole plants, and ABA-insensitive (*abi*) mutants (Koornneef *et al.*, 1984) in which ABA responses are reduced. As shown by the Northern blot data in Figure 5, *ATHB-7* is induced to similar levels by ABA in both the wild-type and the *aba-3* mutant, consistent with the fact that the response to ABA is unaffected by the *aba-3* mutation. Figure 5 also shows ABA induction of *ATHB-7* to wild-type levels in both the *abi2* and *abi3* mutants. Thus, neither the *ABI2* nor the *ABI3* gene products are required for the *ATHB-7* transcription response to ABA. In contrast, induction by ABA at 1 μM was distinctly impaired in the *abi1* mutant. The degree of induction was quantitatively determined in several independent experiments and found to be approximately 30 % of the induction level in the wild-type (data not shown). In addition, the concentration of ABA required for a maximum response in the *abi1* mutant was 10^{-4} M; 100 times higher than for the wild-type (Figure 4b).

Taken together these data indicate that the ABA-induction of *ATHB-7* requires the *ABI1* gene product, and suggests that *ATHB-7* acts downstream to *ABI1* in an ABA-mediated signal transduction pathway for the plant response to water deficit. This hypothesis is supported by data on the effect

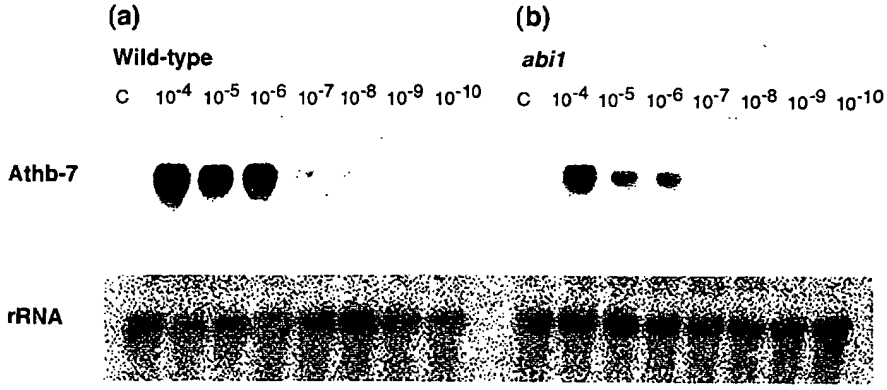

Figure 4. The expression of *ATHB-7* is ABA concentration-dependent.
Northern blot analysis of total RNA, 20 µg per lane, from 10-day-old wild-type (Ler) seedlings (a), or from *abi1* mutant seedlings of *A. thaliana* (b). The seedlings were exposed to different concentrations of ABA (in M as indicated) for 12 h. The filter was hybridized to a 516 bp *Hin*dIII–*Acc*I fragment of the *ATHB-7* clone. The lower panel shows a control hybridization of the same filter to an rRNA probe.

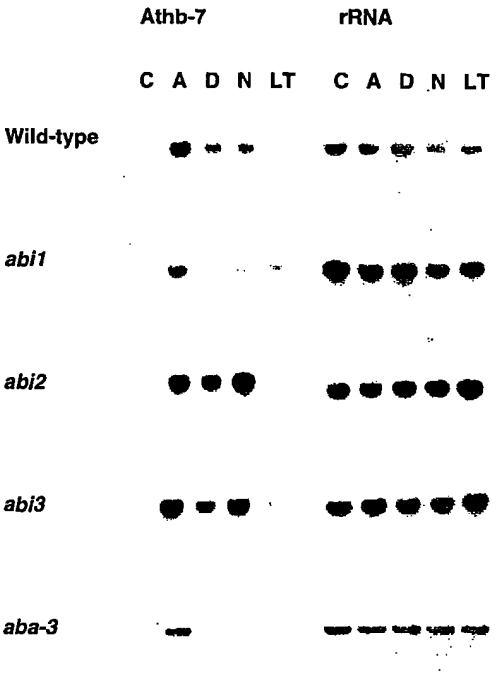

Figure 5. The expression of *ATHB-7* in different *aba* and *abi* mutants in response to ABA, drought, salt and low-temperature treatment.
Northern blot analysis of total RNA samples (10 µg) from 10-day-old wild-type (Ler), ABA-insensitive (*abi*) and ABA-deficient (*aba*) mutant seedlings of *A. thaliana* exposed to 1 µM ABA (A), drought (D), 0.1 M NaCl (N) or low-temperature treatment (LT). C indicates non-treated control plants. The filters were hybridized to a 516 bp *Hin*dIII–*Acc*I fragment of the *ATHB-7* clone. The panels to the right in each case show control hybridizations of the different filters to an rRNA probe.

of water- and osmotic-stress conditions on the expression of *ATHB-7* in the *abi1* mutant, shown in Figure 5. These Northern blot data show that *ATHB-7* transcript levels after water deficit and salt treatments were reduced in *abi1* as compared with the wild-type, the magnitude of the reduction being similar to that observed after ABA treatment. No reduction in the response to either water deficit or salt treatment was observed in the *abi2* or *abi3* mutants. In fact, the induced transcript levels in these two mutants were slightly but reproducibly higher than that observed in the wild-type. These increased transcript levels are possibly attributable to an increased endogenous ABA level in the mutant plantlets, as has previously been documented for seeds of the *abi1, abi2* and *abi3* mutants (Koornneef *et al.*, 1984). In contrast to the wild-type, the *abi1* mutant responded to a low-temperature treatment with a two- to threefold increase in *ATHB-7* transcript level. This response is similar to the *ATHB-7* response to salt treatment in this mutant. A small but reproducible increase in *ATHB-7* transcript in response to low temperature was recorded also in the *abi2* and *abi3* mutants (Figure 5).

Water deficit and osmotic stress induction of ATHB-7 *is mediated by ABA*

The above results suggest that *ATHB-7* induction by water deficit is mediated by a mechanism involving an increase in endogenous ABA levels, as well as the activity of the *ABI1* gene product. To test whether an alteration in endogenous ABA is required for induction we analysed *ATHB-7* expression in the ABA-deficient mutant *aba-3*. Owing to its deficiency in ABA synthesis (Rock and Zeevaart, 1991), the *aba-3* mutant, in contrast to wild-type *Arabidopsis*, does not respond to drought by an increase in ABA. The results (Figure 5) show an increase in *ATHB-7* transcription in the *aba-3* mutant after treatment with exogenous ABA at levels similar to wild-type. No induction, however, could be detected in the *aba-3* mutant after exposure to water deficit, high-salt or low-temperature treatments. Therefore, *ATHB-7* induction by water deficit or osmotic stress is mediated by a mechanism that involves an increase in endogenous ABA.

Discussion

This report describes the first case of a plant homeobox gene which is dependent for its expression on the water conditions of the plant. It also provides the first documentation of a direct functional relationship between a transcription factor and a mutationally defined component of a signal transduction pathway mediating a response to drought in the vegetative part of the plant. Previous work in *Arabidopsis* has identified a transcription factor of the

Myb class which is inducible by water deficit as well as by ABA in a pattern that qualitatively resembles that of *ATHB-7* expression (Urao *et al.*, 1993). This gene, *ATMYB2* differs from *ATHB-7* in that its transcriptional response to ABA is relatively slow; several hours are required for full activation, and the concentration of exogenously added ABA required for a response is considerably higher. *ATHB-7* induction is detectable at concentrations as low as 10^{-8} M ABA and the response is observed within 30 min. As shown by the absence of a drought and salt response in the ABA-deficient *aba* mutant, the drought-induced increase in *ATHB-7* transcription requires endogenous ABA synthesis. Even though the mutant allele used in these experiments, *aba-3*, is a relatively weak allele (Koornneef *et al.*, 1982), the inhibition of induction in the mutant is complete, indicating a strong requirement for ABA synthesis as a mediator of induction. These data strongly indicate that drought and salt-induction of *ATHB-7* is mediated by an increase in endogenous ABA in the plant, and suggest that *ATHB-7* activation is a primary effect, rather than an indirect consequence of drought.

The induction pattern of *ATHB-7* strongly resembles that of some drought-inducible genes previously described from different plants (Gomez *et al.*, 1988; Mundy and Chua, 1990; reviewed by Skriver and Mundy, 1990). Most of these are poorly characterized as regards function. Most likely, though, these genes include ones that take part in the alterations of cellular physiology which result in a tolerance to a reduced water availability, and in the altered growth patterns associated with drought. Since ATHB-7 like ATHB-1 and ATHB-2 (Sessa *et al.*, 1993) is likely to function as a transcription factor, it is an attractive hypothesis that ATHB-7 may be directly active in the transcriptional control of such drought-inducible genes, and thereby constitute the end-point of a signal transduction pathway mediating the response of the plant to water deficit.

The information available on other components of such signal transduction mechanisms is limited to the *ABI* genes, which when mutated confer an ABA-insensitive phenotype to the plant. Our data indicate that *ATHB-7* is a target of the ABA signalling pathway defined by *ABI1* and we propose that *ATHB-7* may function in this signal transduction pathway, downstream to *ABI1*. This suggestion is based on the reduced response of *ATHB-7* to induction conditions in the *abi1* mutant. The recorded induction in *abi1* was approximately 30% of the wild-type response. The fact that *ATHB-7* is still inducible to low levels in the mutant is likely attributable to residual gene activity in this mutant. *ABI1* has been cloned and found to encode a Ca-dependent serine/threonine protein phosphatase (Leung *et al.*, 1994; Meyer *et al.*, 1994). The mutant allele of the gene carries a single base substitution wich causes an alteration in an amino acid located within a domain conserved among S/T phosphatases. The consequences of this mutation for the activity of the enzyme have not been established. The dependence of *ATHB-7* on *ABI1* implies protein phosphorylation to be involved in the transcriptional control of the *ATHB-7* gene. The presence of potential phosphorylation sites for serine/threonine kinases in the sequence of the ATHB-7 protein (Söderman *et al.*, 1994; our unpublished observations) suggests the additional possibility of an influence of ABI1 directly on the activity of the ATHB-7 protein.

The absence of an effect on *ATHB-7* transcription of the *abi3* mutation is consistent with the proposed role for *ATHB-7*, since the phenotypic effects of this mutation are restricted to the developing seed. Our data also show *ATHB-7* transcription to be independent of the activity of the *ABI2* gene, which when mutated causes phenotypic effects that are very similar to those of the *abi1* mutation. Thus, *ABI2* either acts to mediate an ABA response in a second response pathway, independent from that defined by *ABI1*, or functions in the same pathway, downstream to *ATHB-7*. The slightly increased induction levels of *ATHB-7* in response to drought and salt treatments, in both *abi1* and *abi3* mutants as compared with wild-type, are possibly attributable to increased endogenous ABA levels in the mutant plantlets, as has previously been documented for seeds of these mutants (Koornneef *et al.*, 1984).

It is interesting to note that *ATHB-7* transcription in wild-type *Arabidopsis* is unaffected by low-temperature treatment, even though this treatment in *Arabidopsis* as well as in other plant species leads to an increase in endogenous ABA (Chen *et al.*, 1983; Lalk and Dörffling, 1985; Lång *et al.*, 1994). In the *abi1* mutant, on the other hand, *ATHB-7* transcription is induced significantly by the treatment. Thus, it appears that in this mutant, but not in wild-type *Arabidopsis*, *ATHB-7* transcription is influenced by temperature via mechanisms that do not involve ABA. We note the resemblance between this response and that of the ABA-inducible *Arabidopsis* gene *LTI78*, which is inducible by cold, but not by ABA, in the *abi1* mutant (Gilmour and Tomashow, 1991; Nordin *et al.*, 1991).

The documentation of a function of an HD-Zip gene in response to water deficit is interesting, since little is known about the functions of genes of this class. Among the homeobox genes as yet discovered in plants the HD-Zip genes in terms of primary structure of the homeodomain are the most closely related to the animal homeobox genes which act to control essential elements of embryo development. The information available from *Arabidopsis* indicates that the different HD-Zip genes may be active in quite different contexts, but suggests that they also function in the control of developmental processes; *ATHB-8* in the auxin-dependent control of vascular tissue development in the embryo, as well as in the regeneration of vascular tissue after wounding (Baima *et al.*, 1995), and *ATHB-2/HAT4* in the control of plant development in relation to light (Carabelli *et al.*, 1993; Schena *et al.*, 1993), possibly

as a mediator of a phytochrome response. Based on the data presented in this paper we propose that *ATHB-7* may have a similar function in the of control plant growth and development in relation to water availability.

Experimental procedures

Plant material

Arabidopsis thaliana (L.) Heynh., ecotype Columbia was used except in experiments involving the mutants *aba-3, abi1, abi2* and *abi3*, which are all in the Landsberg *erecta* (Ler) ecotype background (Koornneef *et al.*, 1982, 1984). Mutant seeds were kindly provided by the *Arabidopsis* Biological Resource Center (ABRC), Ohio State University, Columbus, Ohio, USA. In experiments involving mutants, the Ler ecotype was used as a wild-type control.

The identity of the *abi1* mutant was confirmed by PCR amplification of part of the *ABI1* gene, followed by sequencing and identification of the molecular alteration described for the *abi1* mutant allele (Leung *et al.*, 1994).

Growth conditions and treatments of plants

Seeds were surface sterilized in a 50% hypochlorite solution containing 0.1% Tween 20 for 10 min and then washed in 70% ethanol for 30 sec and in sterile distilled water several times. The seeds were then either plated on 30 ml solidified (0.8% (w/v) agar) 0.5×MS medium (Murashige and Skoog, 1962; Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 1% sucrose and grown in a culture room at 22°C with a 12 h photoperiod (50 μmol m^{-2} sec^{-1}) or put in 50 ml liquid Gamborg's B5 medium (Gamborg *et al.*, 1968; Duchefa Biochemie) supplemented with 4% sucrose and incubated at 25°C on a rotary platform under continuous illumination (6 μmol m^{-2} sec^{-1}). The plants on solid medium were grown for 10 days and the liquid culture plants for 14 days before treatment.

ABA treatments were performed by addition of ABA (mixed isomers, Sigma) dissolved in Gamborg's B5 medium supplemented with 2% glucose and 0.05% Mes, pH 5.7, to the plant medium. The final concentration of ABA in the medium was 1 μM except when indicated. Control plants were treated with B5 medium without ABA.

NaCl treatments were done by the addition of NaCl to a final concentration of 0.1 M and drought treatments by removal of the lid of the tissue culture plates allowing the plantlets to air-dry. The cold treatment was performed by incubation of the seedlings at +4°C. Treatments with hormones other than ABA were performed by addition of the plant hormones GA3, IAA or kinetin respectively, to liquid cultures to final concentrations of 10 μM. Incubations were for 12 or 72 h or as indicated.

RNA isolation and Northern blot analysis

Total RNA was isolated from plantlets according to the protocol of Chang *et al.* (1993), with the addition of a phenol/chloroform extraction after resuspension of the total RNA pellet. Samples of total RNA (12–30 μg) were subjected to electrophoresis in a 1% agarose gel containing formaldehyde and blotted on to nylon membrane (Hybond-N, Amersham International, Buckinghamshire, UK). The filters were hybridized to a 516 bp *Hin*dIII-*Acc*I fragment of the 3'end of *ATHB-7* (Söderman *et al.*, 1994) which does not contain the conserved homeobox and does not cross-hybridize to other *ATHB*-transcripts (Söderman *et al.*, 1994). The probe was labelled with [α-^{32}P] dCTP (3000 Ci $mmol^{-1}$, Amersham) using the Megaprime DNA Labelling Kit (Amersham). Prehybridization and hybridization were performed at 63°C as described by the filter supplier and the filters were washed at high stringency (0.1×SSPE; 0.1%SDS) at 65°C twice for 5 min. X-ray films were exposed to the filters between intensifying screens.

Equal loading was confirmed by hybridization of the filters to an rRNA probe from Norway spruce (Sundås and Engström, unpublished) and washed at high stringency as above. Quantitative data on hybridization were obtained by use of a BAS 2000 (Fuji) image plate reader.

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Short communication

A new homeodomain-leucine zipper gene from *Arabidopsis thaliana* induced by water stress and abscisic acid treatment

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Key words: abscisic acid, *Arabidopsis*, homeobox, leucine zipper, water stress

Abstract

We report the isolation and characterization of a new homeobox gene from *Arabidopsis thaliana* using a polymerase chain reaction (PCR) cloning strategy. The full-length cDNA, designated *Athb-12*, encodes a protein of 235 amino acids. It contains the conserved DNA binding domain and the leucine zipper motif, characteristic of the homeodomain-leucine zipper family of transcription factors. The deduced amino acid sequence of *Athb-12* shows over 80% identity to the *Arabidopsis* Athb-7 in the homeodomain (82%) and the leucine zipper motif (80%) of the proteins. However, outside the homeodomain and the leucine zipper motif, the homology is significantly lower. RNA analysis identified only one 0.96 kb transcript consistent with the size of *Athb-12* cDNA. The *Athb-12* transcript was detected in stem, leaf, flower and root as well as in seedlings. Treatment with water stress and exogenous abscisic acid (ABA) resulted in the accumulation of *Athb-12* mRNA, similar to that of *Athb-7*. However, the time course of the *Athb-12* response to ABA differed from that of *Athb-7*, suggesting that both genes, in response to ABA, are regulated in different manners. Taken together, these data suggest that *Athb-12* and *Athb-7* are members of a related gene family involved in the plant's response to water stress.

Homeobox genes have been identified in several organisms including various animal species, yeast, fungi, and higher plants. These genes contain a conserved sequence motif, the homeobox, that encodes a sequence-specific DNA-binding domain known as the homeodomain (HD) (for a review, see [3]). In higher plants, a class of the HD genes was first discovered in *Arabidopsis thaliana* [30]. Unlike other classic homeobox proteins, the products of these genes contain a second element that codes for a putative leucine zipper motif, which is closely linked to the carboxy-terminal region of the HD. So far, these proteins termed homeodomain-leucine zipper (HD-Zip) have been identified only in plants such as sunflower [7], carrot [15], soybean [24], tomato [22, 42], rice [21] and *Arabidopsis* [6, 9, 20, 30–33, 40].

The uniqueness of the HD-Zip proteins in plants suggests that these HD-Zip proteins function as a mediator of plant development, for example, coupling of the developmental response to an environmental signal [31]. Several lines of evidence support this notion. Two members of the HD-Zip family, *Arabidopsis Athb-2* and *Athb-4*, show a strong increase in mRNA abundance when treated with far-red-rich light [5, 6]. *Athb-7* and *Athb-8* genes were induced by exogenous plant hormones such as abscisic acid (ABA) and auxin [1, 29, 39]. ABA is involved in the response of plants to environmental stresses (for a review, see [23, 37, 38]. For example, treatment with ABA enhances the resistance of plants to drought [2], salt [17], and cold stress [8]. Several ABA-response genes are induced in response to drought [12], salt [25, 41], and cold stress [16]. A homeobox gene, *Athb-7*, has been reported to be induced by drought as well as ABA [39].

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF001949.

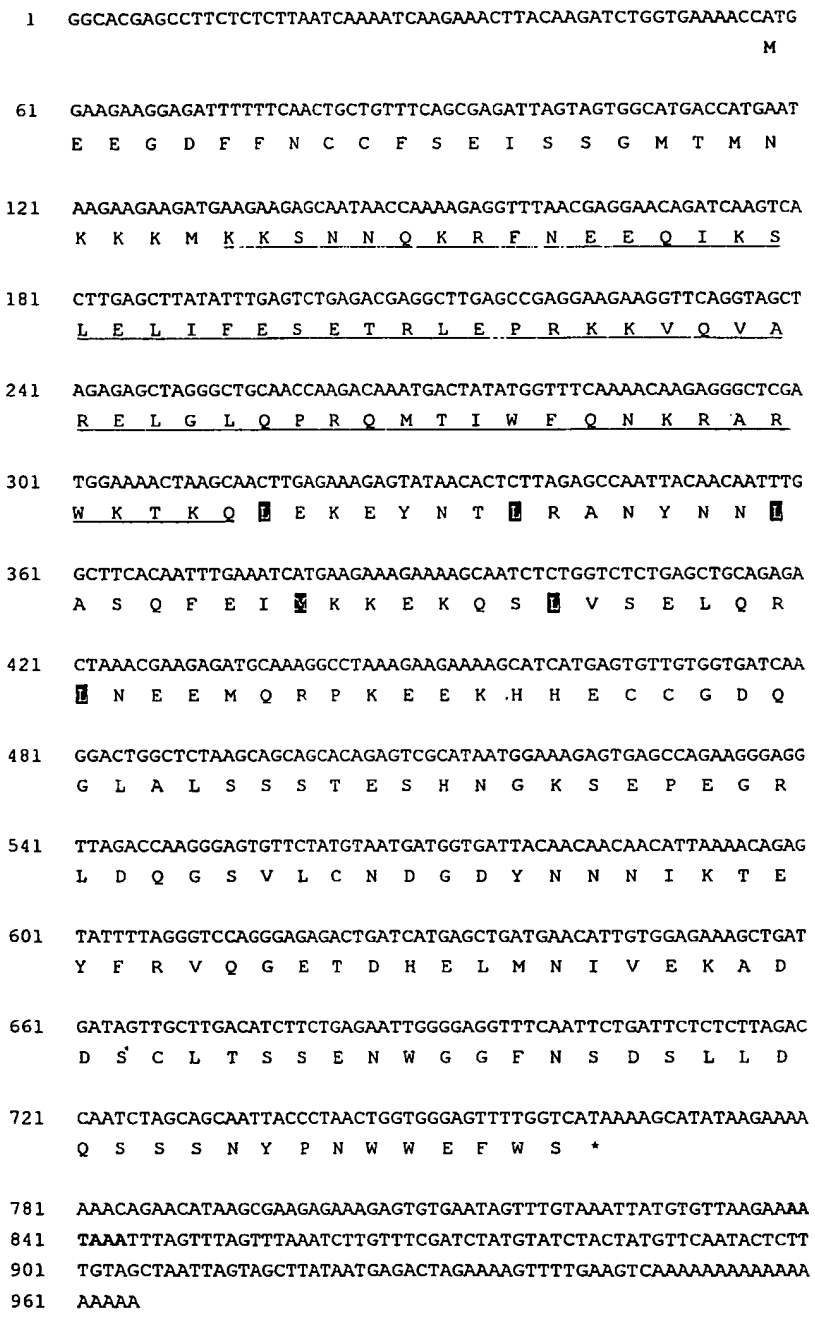

Figure 1. Nucleotide and predicted amino acid sequences of the *Arabidopsis thaliana* cDNA clone encoding Athb-12. The homeodomain is underlined, and the leucine and methionine in the proposed leucine-zipper motif are highlighted in black. The asterisk represents the termination codon. The putative polyadenylation signal is shown in bold-face type. The sequence has been submitted to GenBank/EMBL databases under the accession number AF001949.

Here we report the isolation of an ABA- and water stress-responsive cDNA clone (*Athb-12*) from *A. thaliana*. This gene encodes a new homeodomain-leucine zipper protein. The effect of water stress and ABA on the accumulation of *Athb-12* transcript was studied by northern blot analysis.

PCR isolation of a cDNA fragment that contains a homeobox sequence

To find new homeobox genes from *A. thaliana*, PCR was used to isolate the segments of cDNAs containing homeobox sequences using the *A. thaliana* cDNA library (Stratagene, La jolla, CA) as a template. The universal T3 primer was used as a 5′ primer (forward) for the PCR. A degenerate oligonucleotide, 5′-TTCTGAACCA (G/A/T) AT (A/C) G (C/T) (A/C) A (C/T) (C/T) TG-3′, complementary to the sequences encoding the highly conserved homeodomain helix three was designed based on a comparative analysis of 15 different plant homeodomain DNA sequences selected from GenBank and EMBL databases and used as a 3′ primer (reverse). The PCR reaction mixture contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 1 μM of each primer, 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI) and 2 μl cDNA library suspension containing about 10^8 plaque-forming bacteriophage in a final 50 μl volume. The reaction mixture was heated to 94 °C for 5 min, followed by 40 cycles of amplification at 94 °C for 1 min, 58 ° for 1 min, and 72 ° for 1 min. After the last amplification cycle the samples were incubated at 72 °C for 5 min. The PCR amplification yielded two PCR products, 0.4 kb and 0.3 kb, which were cloned into the pGEM-T vector (Promega) and sequenced. The sequence analysis of both PCR clones showed that 0.4 kb PCR product contains a novel homeobox sequence (not shown). This PCR clone was digested with *Spe*I and *Sac*II and fractionated by electrophoresis. The 0.4 kb PCR DNA fragment was gel-purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and was used as a probe to screen the *Arabidopsis* cDNA library.

Screening of Arabidopsis *cDNA library and isolation of the HD-Zip protein cDNA* Athb-12

A. thaliana whole-plant cDNA library (1×10^6 plaques) was plated at the density of 5×10^4 plaques per plate. A nylon membrane (Hybond-N, Amersham International, Buckinghamshire, UK) was lifted and screened with the labelled 0.4 kb PCR product. The probe was labelled with [α-^{32}P]dCTP using the random labelling kit (Boehringer Mannheim, Mannheim, Germany). The filters were pre-hybridized in a buffer containing 5× SSPE, 5× Denhardt's solution, 0.1% SDS and 0.2 mg/ml denatured salmon sperm DNA for 2 h at 55 °C. Hybridization was carried out overnight at 55 °C

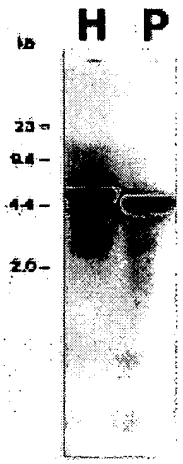

Figure 2. Southern blot analysis of *Arabidopsis thaliana* genomic DNA. *Arabidopsis* genomic DNA (10 μg) was digested with *Hin*dIII (H) or *Pst*I (P). After separation in a 1.0% agarose gel, the digested DNA was transferred onto a nylon membrane and hybridized with the ^{32} P-labelled 3′ end of *Athb-12* cDNA which excluded the homeodomain and leucine zipper region.

in the same solution containing the probe. Filters were washed at 55 °C as follows: two times for 10 min each at 2× SSC/0.1% SDS; once at 1× SSC/0.1% SDS for 10 min; two times for 10 min each at 0.1× SSC/0.1% SDS. X-ray films were exposed to the filters between the intensifying screens. One positive clone hybridized to the 0.4 kb probe was identified and recovered as a plagemid plasmid (designated as pAthb-12) by *in vivo* excision (Uni-ZAP XR Cloning Kit, Stratagene) according to the manufacturer's protocol. The insert of the pAthb-12 cDNA clone was fully sequenced on both strands by the dideoxy chain termination method using Sequenase Version 2.0 Kit (U.S. Biochemicals, Cleveland, OH). Computer-assisted sequence analysis was done with the DNASIS program (Hitachi Software, San Bruno, CA). The cDNA is a 965 bp with a major open reading frame predicted to encode a 235 amino acid polypeptide with a molecular mass of 27 551 Da (Figure 1). The nucleotide sequence around the first ATG codon AACCATGGA (position 54–62) strongly matches the proposed plant translation initiation motif, AACAAUGGC [19]. In addition, the presence of an in-frame TAA stop codon located 37 bp upstream of the 235 amino acid open reading frame suggests that the cDNA is full-length. The open reading frame is followed by 200 bp untranslated region terminating in a poly(A) tail. A putative polyadenylation signal sequence (AATAAA) [14] is present in the 103 bp 5′ end of the poly(A) tail. A search of the NCBI

A. Homeodomain

helix 1 loop helix 2 turn helix3

		Identity(%)
Athb-12	KKSNNQKRFNEEQIKSLELIFESETRLEPRKKVQVARELGLQPRQMTIWFQNKRARWKTKQ	100
Athb-7	HNK---R---D-------MM-------------L----------VA----------S--	82
Athb-6	GL-EKKR-L-IN-V-A--KN--L-NK---ER--KL-Q--------VAV----R--------	59
CHB6	QI-EKKR-LSIN-V-A--KN--V-NK---ER--KL-Q--------VAV----R--------	57
CHB3	QQPEKKR-LKAD--QF--KS--TDNK---E----L-K--------VA-----R-------T	57
Athb-5	TAAEKKR-LGV--V-A--KN--IDNK---ER--KL-Q--------VA-----R--------	57
CHB1	HPPEKKR-LTVD-V-Y--KS--VENK---DR---L-KD-------VA-----R---Y----	54
CHB4	SGGSKKR-LNM--VRT--KS--MGNK---DR-LEL--A-------IA-----R--------	54
Athb-1	QLPEKKR-LTT--VHL--KS--TNKN---ER-T-L-KK-------VAV----R--------	52

B. Leucine zipper motif

Athb-12	L	EKEYNT	L	RANYNN	L	ASQFEI	M	KKEKQS	L	VSELQR	L	100
Athb-7	-	-T---I	-	-Q--D-	-	---- S	L	-----A	-	------	-	80
Athb-6	-	----GD	-	KTQ-DS	-	RHN-DS	L	RRDNE-	-	LQ-ISK	-	36
CHB6	-	-RD-GV	-	K---DS	-	KLKNDT	L	QQ-N--	-	LK-IRE	-	39
CHB3	-	--D-DV	-	QNS--S	-	KADYDN	L	LA--EK	-	KA-VLD	-	36
Athb-5	-	-RD-GV	-	KS-FDA	-	KRNRDS	L	QRDND-	-	LGQIKE	-	25
CHB1	-	--D-DS	-	KEC-DK	-	RDDHDR	L	S--NEK	-	RL-VIL	D	31
CHB4	-	--D-DL	-	KSQFDA	V	KAENDS	L	QSHN-K	-	HAQIMA	-	22
Athb-1	-	-RD-DL	-	KST-DQ	-	L-NYDS	I	VMDNDK	-	R--VTS	-	31

Figure 3. Comparison of the amino acid sequence of the homeodomain and leucine zipper motif of Athb-12 with the corresponding domains of the HD-Zip family members; *Athb-5, -6, -7* [40], *CHB1, 3, 4, 6* [15] and *Athb-1* [30]. Dashes indicate identical amino acids between Athb-12 and other sequences. The highly conserved residues and the four invariant residues in all homeodomains are marked by asterisks (A). The conserved leucine residues in the leucine zipper motif are boxed (B).

database showed that its 5′ end fragment sequence is highly homologous to several *Arabidopsis* expressed sequence tags (ESTs) (accession number N38387, 97% in 315 bp; R30223, 94% in 344 bp; N97195, 97% in 176 bp (identity in nucleotides) [28]). These minor differences in homology suggest that these ESTs may be allelic forms of our cDNA.

Southern blot analysis was used to examine the number of genes encoding Athb-12 in *Arabidopsis*. To avoid cross-hybridization, the 3′ end 550 bp fragment of *Athb-12* cDNA which does not contain the homeodomain and leucine zipper motif region was used as a probe in the Southern blot. The 550 bp fragment was subcloned into pBluescript SK− by removing the 5′ and 410 bp *Pst*I fragment from the cDNA clone, pAthb-12, and self-ligating the plasmid, resulting in plasmid pAthb12-1. The fragment was labelled with [α-^{32}P]dCTP using the random labelling kit (Boehringer Mannheim). Hybridization was done at 55 °C in QuikHyb solution as recommended by the manufacturer (Stratagene). After hybridization overnight, the DNA filters were washed as described for cDNA library screening. Each digestion with the restriction enzymes produced a single signal (Figure 2), suggesting that *Athb-12* gene is a single-copy gene in *A. thaliana*.

Deduced amino acid sequence comparisons

Computer-assisted search through NCBI databases revealed that the deduced protein sequence of *Athb-12* contains a homeodomain (amino acids 26–86) and a leucine zipper motif (amino acids 87–122) (Figure 3). When compared to the available HD-Zip family protein sequences, the Athb-12 protein shares the highest homology with Athb-7 protein: 82%/93% and 80%/89% (identity/similarity) in homeodomain and leucine zipper motif, respectively (Figure 3). We therefore propose that these genes are members of a related gene family. Several related HD-Zip family genes have been identified from *Arabidopsis*. *Arabidopsis Athb-2* and *Athb-4* genes, which have a high amino acid identity (89%) in the HD-Zip motif region, seem to regulate morphological adaptations to changes in light quality [5, 6]. Also, two other related genes, *Arabidopsis KNAT1* and *KNAT2*, which encode proteins with 80% amino acid identity in the homeodomain, may play a role in leaf morphogenesis [18]. Outside the homeodo-

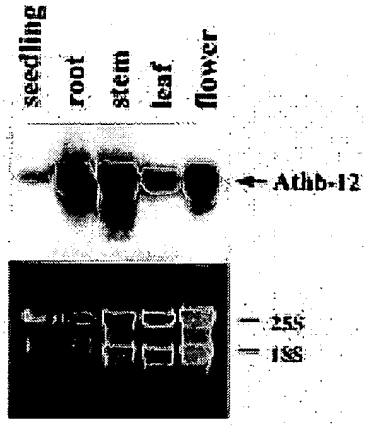

Figure 4. Northern blot analysis of *Athb-12* mRNA expression in *Arabidopsis thaliana*. Total RNA samples of *Arabidopsis thaliana* seedling (3 days old), root, stem, leaf and flower were isolated using RNeasy Plant Total RNA Isolation Kit (Qiagen). RNA was denatured in 1× MOPS buffer (20 mM MOPS, 8 mM sodium acetate, 1 mM EDTA), 50% (v/v) deionized formamide and 2.2 M formaldehyde at 65 °C for 15 min and fractionated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and 1× MOPS buffer. RNA was capillary-blotted in 10× SSC onto a Hybond-N membrane (Amersham). The filter was prehybridized in QuikHyb solution (Stratagene) containing 0.2 mg/ml salmon sperm DNA at 55 °C for 1 h. The probe used was the radiolabeled 550 bp *XbaI-XhoI* fragment of the 3′ end of *Athb*-12 cDNA, which does not contain homeodomain and leucine zipper motifs. Hybridization was carried out at 55 °C. After hybridization overnight, the RNA filters were washed as described for the cDNA library screening in the text. Each lane contains 10 μg of total RNA. The position of *Athb-12* (0.96 kb) is indicated (arrow), as are the positions of the ethidium-stained rRNAs (25S and 18S).

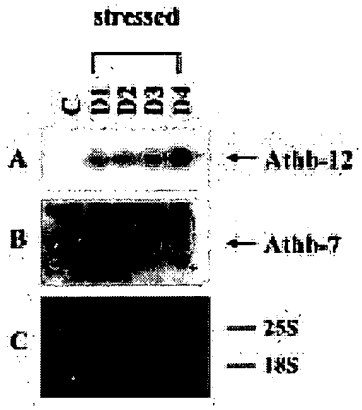

Figure 5. Northern blots showing the effect of water stress on the accumulation of *Athb-12* mRNA. Each lane contains 10 μg of total RNA from *Arabidopsis thaliana* plants raised under standard growth conditions for 14 days and then treated with water-stress. Seeds of *Arabidopsis thaliana* (columbia ecotype) were surface-sterilized in a 70% ethanol for 15 s and then in 10% chlorox solution for 10 min, followed by at least five rinses in sterile distilled water. The seeds were plated on 30 ml solidified 0.8% agar plates containing 0.5× MS medium [27] supplemented with 1.5% sucrose. They were grown in a culture room at 25 °C with a 16 h photoperiod for 14 days before water stress treatment. Isolation of total RNA from whole plants and northern blot were done as described for Figure 4. C represents untreated control plants. A. RNAs from control plants or plants harvested 1 (D1), 2 (D2), 3 (D3), and 4 (D4) days after exposure to water stress were probed with the 3′-specific probe of *Athb-12* cDNA. B. Blot A was reprobed with a 3′-specific probe of *Athb-7* after removing the *Athb-12* probe. The *Athb-7* cDNA fragment, which does not contain homeodomain and leucine zipper motifs, was amplified by using the *Athb-7*-specific primers (5′ primer, 5′-AAAGAGGCGACGCAAAAGAAGA′-3 and 3′, primer, 5′-CTACTTAGCTACAAAGCATGACGAG-3′). The PCR products were subcloned into pGEM-T vector (Promega) and verified by sequencing analysis. The positions of the *Athb-12* (0.96 kb) and *Athb-7* (1.17 kb) are indicated (arrow). C. The photograph of ethidium bromide-stained rRNAs (25S and 18S).

main and leucine zipper motif, the homology between Athb-12 and Athb-7 proteins is significantly lower.

Figure 3 shows the alignment of the homeodomain and leucine zipper motif of Athb-12 with the corresponding amino acid sequences of the published plant sequences. The amino acid sequences of the HDs are more similar to each other than to the leucine zipper motifs. This feature has also been usually observed in other HD-Zip family proteins. All of these proteins showing a high homology to Athb-12 belong to the class I HD-Zip family. Based on their sequence homology, the HD-Zip proteins have been tentatively grouped into four different families, named HD-Zip I, II, III and IV [21, 35]. The homeodomain in Athb-12 possesses the four invariant amino acid residues found in all the homeodomains of higher eukaryotes [34] as well as five out of the eight highly conserved residues [10].

The leucine zipper motif lies adjacent to the C-terminal side of the homeodomain of Athb-12. The leucine zipper motif is known to form an amphipathic α-helix with a series of leucine residues spaced by exactly seven amino acid residues and to be responsible for dimerization to juxtapose a pair of target DNA contacting surface [4]. Recently, it has been shown that the HD-Zip family proteins Athb-1 and Athb-2 recognize dyad-symmetric DNA sequences as homodimers formed via dimerization of the leucine zipper [36]. Therefore, the presence of a homeodomain and a leucine zipper motif in the Athb-12 suggests that *Athb-12* encodes a DNA-binding protein which may also exist as a dimer.

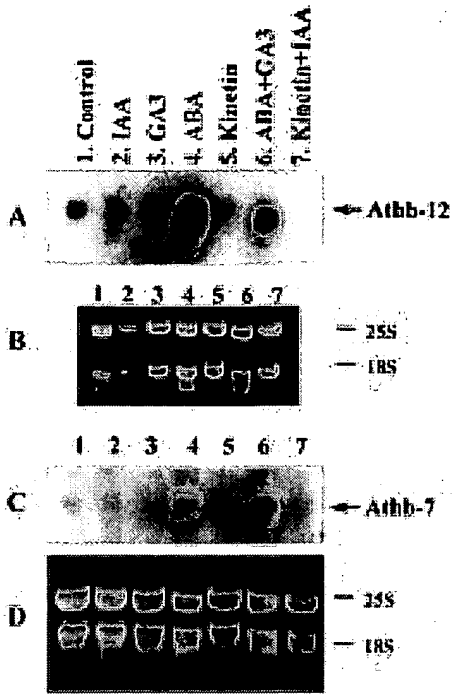

Figure 6. Northern blot analysis showing the effect of exogenously applied plant hormones on the accumulation of *Athb-12* and *Athb-7* mRNAs. The sterilized seeds of *Arabidopsis thaliana* (Columbia ecotype) were placed in 50 ml liquid MS medium supplemented with 1.5% sucrose and grown on a rotary platform for 14 days before hormone treatment. Treatments with hormones were performed by addition of the plant hormones abscisic acid (ABA), gibberellic acid (GA_3), indole-acetic acid (IAA), kinetin or a combination of ABA + GA_3 or IAA + kinetin to liquid cultures to the final concentration of 10 μM. Samples were harvested after treatment for 72 h. Isolation of total RNA from seedlings and northern blot were done as described for Figure 4. Each lane contains 10 μg of total RNA. Lane 1 is a MS medium-treated control sample without hormone treatments. A. The blot was hybridized with the 3′-specific probe of *Athb-12* cDNA. C. The blot was hybridized with the 3′-specific probe of *Athb-7* cDNA. The positions of the *Athb-12* and *Athb-7* are indicated (arrow). B and D. The photographs of ethidium bromide-stained rRNAs (25S and 18S).

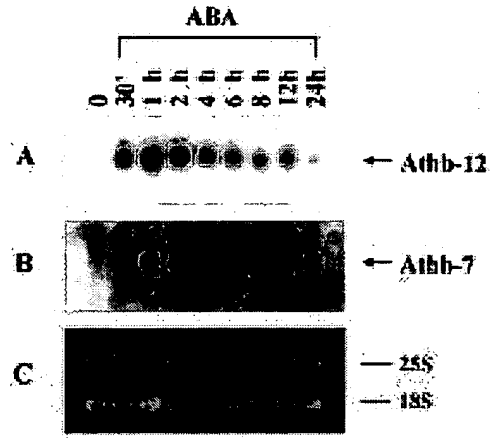

Figure 7. The time courses of accumulation of the *Athb-12* and *Athb-7* mRNAs in response to ABA. A 10 μg portion of total RNA samples isolated from *Arabidopsis* seedling grown in liquid cultures for 14 days and then treated with ABA was used in each lane. The final concentration of 10 μM ABA was added into the liquid medium and samples were harvested at the indicated times (0 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h) after treatment with ABA. Isolation of total RNA from seedlings and northern blot were done as described for Figure 4. A. The blot was hybridized with the 3′-specific probe of *Athb-12* cDNA B. The blot in A was reprobed with a 3′-specific probe of *Athb-7* after removing the *Athb-12* probe. The positions of the *Athb-12* and *Athb-7* are indicated (arrow). C. The photograph of ethidium bromide-stained rRNAs (25S and 18S).

Expression of the Athb-12 *gene in different organs*

To determine the expression pattern and size of *Athb-12* transcript in different organs of *Arabidopsis*, we conducted a RNA gel blot hybridization analysis using the 3′-specific cDNA probe of *Athb-12*. This probe does not contain the homeodomain and leucine zipper region. Figure 4 shows that a single band of ca. 0.96 kb transcript is detected in mRNAs isolated from all organs such as root, stem, leaf and flower as well as seedling. The *Athb-7* gene has also been reported to be expressed in all organs of plant [40]. The estimated size of the *Athb-12* transcript closely agrees with the size of the *Athb-12* cDNA.

Effect of water stress and ABA on the accumulation of Athb-12 *mRNA*

Athb-7 mRNA is induced by environmental stress such as drought and exogenous ABA [39]. Since the similar expression pattern and the high amino acid sequence homology between Athb-12 and Athb-7 observed, we tested whether Athb-12 is induced by water stress. Exposure to water stress was done by removing the lid of the tissue culture plates and letting the plantlets to air-dry for the times indicated. The level of *Athb-12* mRNA increased in response to water stress under our experimental conditions (Figure 5A). The higher level of accumulation was detected after 4 days of treatment by air drying (Figure 5A). When the plants were treated for 5 days of air drying, they were too dry to isolate RNA samples (not shown). The conditions of water stress treatment were tested by reprobing the same RNA blots with a cDNA fragment derived from the *Athb-7* gene, described as a drought-inducible gene, after removing the *Athb-12* probe. The level of *Athb-7*

transcript also increased in response to these water-stress conditions (Figure 5B). These results strongly suggest that the accumulation of *Athb-12* mRNA is regulated by water status of the plant.

Many drought-inducible genes have been shown to be responsive to exogenous ABA [11, 12, 13, 26, 43]. We examined the effect of exogenous ABA on the expression of *Athb-12* by RNA gel blot analysis. The expression level of *Athb-12* increased in response to exogenous ABA, as compared to the MS medium-treated control without ABA (Figure 6A, lanes 1 and 4). We also examined the effects of other plant hormones, gibberellic acid (GA3), indoleacetic acid (IAA) and the cytokinin kinetin, on the expression of *Athb-12*. Treatment with these hormones did not result in a significant change in the expression level of *Athb-12* under our experimental conditions (Figure 6A, lanes 2, 3 and 5). The *Athb-12* mRNA level also increased by the combination of ABA and GA_3, but not by the combination of IAA and kinetin (Figure 6A, lanes 6 and 7). To establish whether the concentrations and activities of the hormones are effective under our experimental conditions, the RNA gel blot was stripped and reprobed with a cDNA fragment derived from *Athb-7* gene described as an ABA-inducible gene. As we expected, the *Athb-7* transcript was induced by ABA but not by other hormones, consistent with that of *Athb-12* (Figure 6C).

We further examined the time course of accumulation of *Athb-12* mRNA in response to exogenous ABA. The expression of *Athb-12* transcript was strongly induced within 30 min after ABA treatment (Figure 7A). The transcript level reached a maximum at 1 h and then gradually decreased until 24 h (Figure 7A). To compare the time course of the *Athb-12* and *Athb-7* responses to ABA, the same RNA blots were reprobed with *Athb-7* gene. The induction pattern of *Athb-7* in response to ABA differed from that of *Athb-12* (Figure 7A and B). A significantly increased level of *Athb-7* mRNA was observed after 1 h of ABA treatment and reached a maximum at 4 h (Figure 7B). These results suggest that both genes in response to ABA are regulated in different manners.

Taken together we propose that Athb-12 and Athb-7 proteins are involved in the plant's response to water stress in different manners. Most of the homeobox genes identified in plants have been reported to be responsible for the development of plant. However, Athb-12 appears to be involved in environmental stress signaling such as water stress. It can serve as a useful model system to study the functions of homeobox genes in the plant's responses to environmental stresses.

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Kumho Life and Environmental Science Laboratory Publication No. 9.

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The homeobox genes *ATHB12* and *ATHB7* encode potential regulators of growth in response to water deficit in *Arabidopsis*

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Abstract

The *Arabidopsis thaliana* homeodomain leucine-zipper gene *ATHB7*, which is active specifically under water deficit conditions, is proposed to act as a negative regulator of growth (Söderman *et al.*, 1996, Plant J. 10: 375–381; Hjellström *et al.*, 2003, Plant Cell Environ 26: 1127–1136). In this report we demonstrate that the paralogous gene, *ATHB12*, has a similar expression pattern and function. *ATHB12*, like *ATHB7*, was up-regulated during water deficit conditions, the up-regulation being dependent on abscisic acid (ABA) and on the activity of the Ser/Thr phosphatases ABI1 and ABI2. Plants that are mutant for *ATHB12*, as a result of T-DNA insertions in the *ATHB12* gene, showed a reduced sensitivity to ABA in root elongation assays, whereas transgenic *Arabidopsis* plants expressing *ATHB12* and/or *ATHB7* as driven by the CaMV 35S promoter were hypersensitive in this response compared to wild-type. High-level expression of either gene also resulted in a delay in inflorescence stem elongation growth and caused plants to develop rosette leaves with a more rounded shape, shorter petioles, and increased branching of the inflorescence stem. Transgenic *Arabidopsis* plants expressing the reporter gene *uidA* under the control of the *ATHB12* promoter showed marker gene activity in axillary shoot primordia, lateral root primordia, inflorescence stems and in flower organs. Treatment of plants with ABA or water deficit conditions caused the activity of *ATHB12* to increase in the inflorescence stem, the flower organs and the leaves, and to expand into the vasculature of roots and the differentiation/elongation zone of root tips. Taken together, these results indicate that ATHB12 and ATHB7 act to mediate a growth response to water deficit by similar mechanisms.

Abbreviations: ABRE, ABA responsive element; ATHB, *Arabidopsis thaliana* homeobox; CE, coupling element; HDZip, homeodomain leucine-zipper; PP2C, protein phosphatase 2C

Introduction

When grown under water deficit conditions plants respond at the molecular, cellular and physiological level to reduce water loss and develop long-term tolerance to low water availability. Some responses, like the closure of stomata to minimise transpiration, are fast, whereas others, like an overall growth reduction, are slower (Himmelbach *et al.*, 1998). Plants are exposed to decreased water availability when subjected to drought, cold or high salinity. These conditions cause an increase in the endogenous levels of abscisic acid (ABA), a crucial growth-inhibiting hormone, which controls vegetative growth in response to environmental stress (associated with high levels of endogenous ABA) and acts as a growth promoter in the absence of stress (associated with low levels of

endogenous ABA (Himmelbach *et al.*, 1998; Cheng *et al.*, 2002). ABA also acts as a developmental signal to regulate diverse processes such as seed maturation, seed germination and the general response of vegetative tissues to environmental stress conditions (Zeevaart and Creelman, 1988; McCarty, 1995; Leung *et al.*, 1998).

A number of mutants have identified genetic loci involved in ABA biosynthesis and perception. Their pleiotropic phenotypes demonstrate the importance of ABA and the complexity of the regulatory networks in which ABA is involved (for recent reviews see Finkelstein *et al.*, 2002; Fedoroff, 2002). In *Arabidopsis* the ABA insensitive mutants *abi1, abi2, abi3, abi4* and *abi5* germinate on medium with ABA at concentrations that are inhibitory to wild-type (Kornneef *et al.*, 1984; Finkelstein, 1994). The *abi3*, *abi4* and *abi5* mutants have reduced ABA responses that are mainly restricted to seed development, whereas the *abi1* and *abi2* mutants affect ABA sensitivity both in the seed and in the vegetative parts of the plant. *ABI1* and *ABI2* encode related serine/threonine protein phosphatases (PP2C; Leung *et al.*, 1994, 1997; Meyer *et al.*, 1994; Bertauche *et al.*, 1996) suggested to act as negative regulators of ABA signalling (Gosti *et al.*, 1999; Merlot *et al.*, 2001). The dominant *abi1-1* and *abi2-1* mutants, with base pair substitutions at equivalent positions in the PP2C encoding domains of each gene, exhibit similar phenotypic alterations, including reduced ABA sensitivity during seed germination and root elongation, reduced seed dormancy and an altered ABA response of stomata, as compared to wild-type (Koornneef *et al.*, 1984; Finkelstein and Sommerville, 1990; Leung *et al.*, 1997). Putative *abi1* and *abi2* loss-of-function mutations, referred to as *abi1-1R1–abi1-1R7* and *abi2-1R1*, are recessive intragenic suppressor mutations, and cause plants to be ABA hypersensitive (Gosti *et al.*, 1999; Merlot *et al.*, 2001). By similar screens, mutants with enhanced sensitivity to ABA, have also been identified (reviewed by Finkelstein *et al.*, 2002).

Among the drought, cold, high salinity and/or ABA induced genes that encode transcription factors, a few have been shown to bind to *cis*-acting elements of stress induced genes. This group includes members of the drought/cold response element binding protein (DREB/CBF) class (Stockinger *et al.*, 1997; Liu *et al.*, 1998) of the AP2 family; the ABA response element binding protein class (AREB/ABF) of the bZIP family (Choi *et al.*, 2000; Uno *et al.*, 2000; Kang *et al.*, 2002); and the MYB and MYC families (Abe *et al.*, 1997, 2003; Gilmour *et al.*, 1998; Chinnusamy *et al.*, 2003). Other stress induced transcription factor genes have also been identified (Seki *et al.*, 2002a, b). This group includes genes that encode homeodomain leucine-zipper (HDZip) proteins.

The HDZip gene family in *Arabidopsis* includes 42 genes (Arabidopsis Genome Initiative, 2000). HDZip proteins interact as dimers by their leucine-zippers domains, and bind DNA sequence specifically via their homeodomains (Sessa *et al.*, 1993; Aoyama *et al.*, 1995; Meijer *et al.*, 1997; Frank *et al.*, 1998; Johannesson *et al.*, 2001). Based on sequence criteria, the HDZip proteins have been grouped into four classes (Sessa *et al.*, 1994). Functional data available on a subset of the class I and II genes have shown a number of them to be involved in developmental reprogramming in response to changes in environmental conditions. The class II genes *ATHB2* and *ATHB4* are essential for the shade avoidance response (Carabelli *et al.*, 1996; Steindler *et al.*, 1999; Ohgishi *et al.*, 2001). The class I gene *ATHB16* is thought to mediate blue-light-responses (Wang *et al.*, 2003) and *ATHB13* is suggested to have a role in sucrose signalling (Hanson *et al.*, 2001). The class I genes *ATHB5, ATHB6* and *ATHB7* are suggested to regulate aspects of the plant response to ABA. The expression pattern of *ATHB5* in seedlings is altered in response to ABA treatment, and transgenic *Arabidopsis* plants that express *ATHB5* at high levels have an enhanced sensitivity to the inhibitory effects of ABA on germination and seedling growth (Johannesson *et al.*, 2003). *ATHB6, ATHB7* and *ATHB12* are implicated in the plant response to water deficit as deduced from their transcriptional induction by water deficit conditions or ABA treatment (Söderman *et al.*, 1996 and 1999; Lee and Chun, 1998). High-level expression of *ATHB6* confers a reduced ABA sensitivity to germinating seeds and stomata in transgenic *Arabidopsis* plants (Himmelbach *et al.*, 2002). The growth pattern of transgenic plants with high-level expression of *ATHB7* during normal growth conditions, mimics the phenotype of wild-type plants grown under water limiting conditions, with reduced elongation of the

inflorescence stem and rosette leaves (Hjellström *et al.*, 2003). The phenotypes of transgenic plants with constitutive high-level expression of *ATHB6* or *ATHB7* support the notion that the genes have water deficit response functions.

In this report we describe the functional characterisation of *ATHB12* in *Arabidopsis*. *ATHB12* is closely related to *ATHB7*, the two genes sharing over 80% identity in the deduced amino acid sequence of their HDZip motives and a common intron–exon organisation, distinct from other members of the HDZip class I. In contrast to other class I HDZip proteins, *ATHB12* and *ATHB7* do not bind *in vitro* to the class I consensus binding sequence CAATNATTG in electrophoretic mobility shift assays (Johannesson *et al.*, 2001). Our data show that *ATHB12* and *ATHB7* both act as growth regulators in response to water deficit conditions by similar mechanisms.

Materials and methods

Growth conditions and treatments of plants

Seeds were surface sterilised in 35% chlorine and 0.2 % Tween 20 (v/v), washed in sterile distilled water several times and plated on 0.8% (w/v) agar, 0.5 × MS medium (Murashige and Skoog, 1962; Duchefa Biochemie B. V., Haarlem, the Netherlands) supplemented with 1% sucrose in petri dishes. Seeds were cold treated for 2 days at 4 °C in darkness, transferred to continuous cool light (70 μEm^2s) at 21 °C, germinated and grown for 10 days. Plants to be studied at later stages were transferred to a mixture of soil and vermiculite (1:1) and grown with a photoperiod of 8 h darkness/16 h light at 100 μEm^2s.

For ABA treatments, seeds were germinated and grown according to Gosti *et al.* (1999) on sucrose free medium containing 0.5 × MS (Murashige and Skoog, 1962). ABA (mixed isomers; Sigma) was diluted from a 50 mM stock solution prepared in 70% ethanol and equivalent volumes of ethanol were included in all samples.

Seedlings exposed to water deficit conditions were left on the growth plate with the lid off for approximately 24 h. In experiments with adult plants seeds were germinated and grown for 10 days on 0.5 × MS supplemented with 1% sucrose and transferred to soil. Water deficit conditions were induced by withholding of water after transfer to soil.

For test of other stress conditions, 10–14-day-old seedlings were exposed to 50μM ACC (aminocyclopropane-carboxylic acid) for 6 h or low temperature (4 °C) for 12 h. Three weeks old plants were completely flooded for 1, 4 or 8 h. Adult plants were mechanically wounded and analysed after 24 h, innoculated with *Pseudomonas syringae* and analysed after 1, 2, 3 or 4 h, treated with 1 mM SA (salicylic acid) for 12 h or with 150 μM MeJA (methyl jasmonate) for 48 h.

For root elongation assays, seeds were germinated and grown on vertical plates for four days on ABA-free medium in continuous light and thereafter transferred to vertical plates supplemented with ABA at 0, 0.1, 1, 5, 10, 50 or 100μM. After four additional days, root growth was measured.

Germination was scored as radicle emergence after two days in continuous light on sucrose free 0.5 × MS medium containing 0, 0.25, 0.5, 0.75, 1 or 2 μM ABA.

The time of flowering was recorded by a stereomicroscope, as the time point of appearance of flower meristems.

RNA gel blot analysis

Total RNA was isolated according to the protocols of Chang *et al.* (1993) or Verwoerd *et al.* (1989). About 10 μg samples of total RNA (20 μg in the experiments shown in Figure 1A) were loaded onto 1% agarose gels containing formaldehyde, subjected to electrophoresis and blotted to nylon membranes (Hybond-XL, Amersham Pharmacia Biotech, Uppsala Sweden). These membranes were pre-hybridised, hybridised, washed and stripped according to the manufacturer's instructions. Probes were labelled with [α-^{32}P] dCTP (3000 Ci/mmol, Amersham) using the Megaprime DNA Labelling Kit (Amersham). The final washes were conducted in 0.1 × SSC, 0.1% (w/v) SDS at 65 °C. The *ATHB12* probe used was a 350 bp PCR fragment amplified by use of the following primers: 5′-CGCGGATCCGACTAAACGAAGAGATGCAAAGGC-3′ and 5′-CGCGGATCCGCTTTTATGACCAAAACTCCCACC-3′. The *ATHB7* probe was a 516 bp *Hind*II-*Acc*I fragment from the *ATHB7* cDNA. A *uidA* specific probe was PCR amplified with the

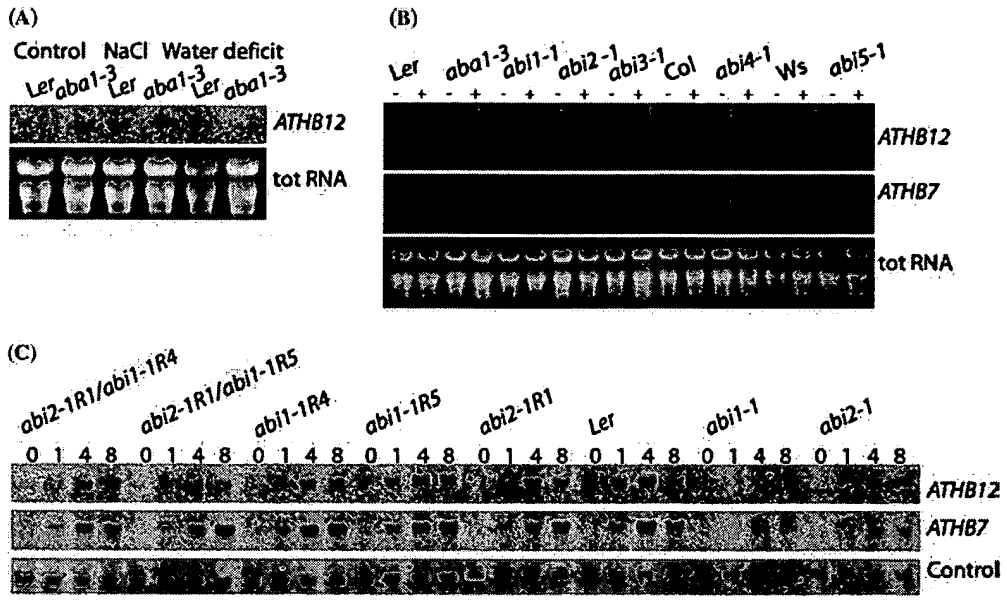


Figure 1. Water deficit induction of *ATHB12* depends on abscisic acid, ABI1 and ABI2. RNA gel blot analyses of RNA samples extracted from10-day-old: (A) L*er* (wt) and ABA deficient *aba1-3* seedlings treated with control solution, 100 mM NaCl for four hours or exposed to water deficit conditions that resulted in approximately 50% water loss; (B) L*er*, *aba1-3*, *abi1-1*, *abi2-1*, *abi3-1* (all in L*er* background), Col (wild-type background to *abi4-1*), *abi4-1*, Ws (wild-type background to *abi5-1*) and *abi5-1* seedlings non-treated (−) or treated with 10 μM ABA for four hours (+); (C) *abi1-1R4*/*abi2-1R1*, *abi1-1R5*/*abi2-1R1*, *abi1-1R4*, *abi1-1R5*, *abi2-1R1*, L*er* (wt), *abi1-1* and *abi2-1* exposed to 10 μM ABA for 0, 1, 4 or 8 h as indicated. The gels were blotted to filters that were subjected to hybridisation of gene specific probes corresponding to *ATHB12*, *ATHB7* and control probes, consecutively. The bottom panels in A and B show ethidium bromide stained RNA gels before blotting, as a control for equal loading of RNA. Interpretations from results in C are based on quantitative data normalised to the hybridisation signal from the control probe and from results from repeated experiments. The control probe was an 536 bp *Eco*RI fragment of the ribosomal gene *AtL18* (Baima *et al.*, 1995).

following primers: 5′-TGATGTCAGCGTTGAACTGCG-3′ and 5′-TCAGCGTAAGGGTAATGCGAGG-3′. An *Eco*RI fragment corresponding to 563 bp of the ribosomal gene *AtL18* was used as probe in control hybridisations (Baima *et al.*, 1995). The hybridisation results were visualised and quantified using a BAS2000 Image Plate Reader (Fuji Photo Film, Tokyo, Japan).

Screening T-DNA insertion libraries for athb12-mutants

An *athb12-1* mutant was isolated, from the CD6-7 DNA pool by use of PCR (Arabidopsis Biological Resource Center, ABRC, Ohio State University, Columbus, USA), with the use of the following primers: T-DNA left border; 5′-CATCGGTCTCAATGCAAAAGGGGAACG-3′; T-DNA right border; 5′-ACGTCGTGATGGGAAAACCTGGCGTTAC-3′; *ATHB12* forward primer; 5′-GCTGTTTCAGCGAGATTAGTAGTG-3′; *ATHB12* reverse primer; 5′-AGCAATTACCCTAACTGGTG-3′.

The *athb12-2* and *-4* mutants were isolated from the Wisconsin activation tagging T-DNA BASTA-collections and *athb12-3* from the Wisconsin ALPHA-collection (http://www.biotech.wisc.edu/) according to Krysan *et al.* (1999) using the T-DNA primer JL202; 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′, the *ATHB12* specific forward; 5′-AAACTGAAAGAGACATGAGCCAATTCGTG-3′ and reverse; 5′-CAAACTATTCACACTCTTTCTCTTCGCTT-3′ primers.

Using Southern blot, positive PCR products that hybridised to the *ATHB12* cDNA were identified. The PCR products were cloned and sequenced to determine the position of the T-DNA insertion. The corresponding *athb12*-mutant plants were identified according to the protocol described by Krysan *et al.* (1999). Plant lines showing 100% growth on 50 μg/ml kanamycin or

8 mg/ml Basta were back-crossed to wild-type and F2 plants with 3:1 segregation on selective medium were isolated for propagation of homozygous mutant plants and further analyses.

Generation of transgenic plants with high-level expression of ATHB12

An *ATHB12* cDNA fragment was isolated from EST 157L22T7 (ABRC), cut with *Bam*HI and *Bgl*II and ligated in the sense orientation into the *Bam*HI site downstream of the CaMV 35S promoter in pHTT202 vector (Elomaa *et al.*, 1993), confirmed by sequencing and transferred to *Agrobacterium tumefaciens* strain C58::pGV2260 by triparental mating.

Transformation of *Arabidopsis thaliana* ecotype Wassilevskija (Ws) and *35S::ATHB7* sense transformants in Ws background (the M4 line described by Hjellström *et al.*, 2003) was performed using an infiltration technique (Clough and Bent, 1998). Independent lines segregating 1:3 on selective medium in the F2 generation were propagated to give homozygous seeds.

Generation of ATHB12 *promoter*-uidA *fusion constructs, and GUS assays*

Approximately 240 000 plaques from an *Arabidopsis* ecotype Columbia (Col) genomic library, constructed in λEMBL3 SP6/T7 were screened according to standard protocols (Sambrook *et al.*, 1989) with a gene specific *Pst*I-*Bam*HI fragment of the *ATHB12* cDNA as probe. A 3.5 kb *Sal*I genomic DNA fragment was subcloned into the pBluescript SK+ vector (Stratagene, La Jolla, CA) from which a 3.2 kb *Hind*III-*Pst*I fragment was isolated and fused in frame with the *uidA* gene (Jeffersson *et al.*, 1987) in the pBI101.1 binary vector (Clontech, Palo Alto, CA, USA), resulting in a translational fusion between *ATHB12* and the reporter gene. Correct identity of the construct was confirmed by sequencing using an ABI automated sequencer (Perkin-Elmer, Norwalk, CT). The plasmid was transferred to *A. tumefaciens* strain GV3101, with the helper plasmid pMP90 by triparental mating and to *Arabidopsis* ecotype Ws by use of vacuum infiltration (Clough and Bent, 1998). Nine independent lines that segregated 3:1 on 50 μg/ml kanamycin in the T2 generation were generated. 10-day-old ABA treated homozygous T3 seedlings from these nine lines and flowers from plants grown under normal conditions were assayed for GUS activity *in situ*. Three representative lines were selected for detailed histochemical analyses with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) as described by Jefferson *et al.* (1987). Stained tissues were cleared in ethanol or essentially according to the protocol of Berleth and Jürgens (1993); chloral hydrate being substituted by 8 M NaOH for incubation over night, followed by a treatment with 0.1 M potassium phosphate buffer, pH 7.5. GUS stained plants were fixed in 4% formaldehyde and 0.1 M PIPES buffer according to Di Laurenzio *et al.* (1996), embedded in epoxiplast (TAAB 812) and sectioned to 2 μm. The plants were photographed with interference contrast microscopy or normal light stereomicroscopy.

Results

Water deficit induced transcription of ATHB12 *is dependent on abscisic acid, ABI1 and ABI2*

Arabidopsis seedlings, when exposed to water deficit stress conditions or high salinity, show a distinct increase in the level of *ATHB12* transcript (Figure 1A, Lee and Chun, 1998). Similar or higher increases in transcript levels are observed in seedlings treated with ABA (Figure 1B, Lee and Chun, 1998) whereas treatment with MeJA, SA, ACC or exposure to chilling, flooding, inoculation of the pathogen *P. syringae* or wounding did not affect *ATHB12* transcript levels (data not shown). The specificity in the transcriptional response suggests that *ATHB12* has a role in ABA regulated water stress responses.

In the ABA-deficient mutant, *aba1-3*, the *ATHB12* transcript level in plants exposed to water deficit or NaCl treatment did not differ from the non-treated control plants (Figure 1A). In contrast, *aba1-3* plants responded to exogenously applied ABA by an increase in *ATHB12* transcript levels similar to that in the wild-type (Figure 1B). These findings show that the transcriptional response of *ATHB12* to water deficit is dependent on ABA.

As shown in Figure 1B the transcriptional response of *ATHB12* to ABA was reduced in the ABA response mutants *abi1-1* and *abi2-1*, whereas

the response in the *abi3-1*, *abi4-1* and *abi5-1* mutants did not differ reproducibly from the wild-type response. Figure 1B also demonstrates that the transcriptional response of *ATHB7* to ABA in the ABA synthesis and the ABA response mutants, was very similar to that of *ATHB12*. The ABA induction of *ATHB7* was reduced in *abi1-1* (Figure 1B) as well as in *abi2-1*. The reduction in the transcriptinal response of both genes was more pronounced in *abi1-1* than in *abi2-1*, and the reduction in the *ATHB12* response was more severe than that of *ATHB7* in both mutants.

The ABA induced increases in transcript levels of both *ATHB12* and *ATHB7* in the wild-type were initiated within one hour and reached a maximum at 4 h after the ABA application (Figure 1C). In *abi1-1* and *abi2-1* the ABA induction of *ATHB12* and *ATHB7* showed similar time-courses as in the wild-type, but the levels of induction were reduced. In the putative loss off function *abi1* and *abi2* mutants; *abi1-1R4*, *abi1-1R5*, *abi2-1R1*, *abi1-1R4/abi2-1R1* and *abi1-1R5/abi2-1R1* the time course of *ATHB12* and *ATHB7* induction was also similar to wild-type. The level of induction of *ATHB12* in these mutant varied between experiments, but was consistently lower than in the wild-type. In the single *abi1-1R4*, *abi1-1R5* and *abi2-1R1* mutants the *ATHB12* mRNA levels were reduced by 15–50% and in the corresponding double mutants by 20–65% as compared to wild-type. The transcriptional response of *ATHB7* to ABA, in these mutants plants was similar to that of *ATHB12*, except that the reduction in the response, as compared to wild-type, was less pronounced (Figure 1C).

athb12 *T-DNA insertion mutant plants show a decreased sensitivity to ABA in root elongation assays*

Screens of *Arabidopsis* T-DNA insertion mutant collections for mutants in *ATHB12* resulted in four T-DNA insertion lines, referred to as *athb12-1*, *athb12-2*, *athb12-3* and *athb12-4*. *athb12-1* carries a T-DNA insert (Figure 2A) 900 nucleotides downstream of the transcription start, in the coding sequence at a position corresponding to the C-terminal part of the deduced protein, up-stream of the putative activation domain (Lee *et al.*, 2001). *athb12-1* encodes an *ATHB12* transcript, which is shorter than the wild-type transcript (Figure 2B), consistent with the position of the T-DNA insert. This transcript, like the wild-type transcript was inducible by ABA (Figure 2B). The *athb12-2*, *-3* and *-4* mutants harbour T-DNA insertions positioned 178, 277 and 413 nucleotides upstream of the transcription start of *ATHB12* (Figure 2A). As shown in Figure 2B no *ATHB12* transcript can be detected in RNA gel blot analyses of *athb12-2* plants treated with 50 μM ABA or non-treated plants. ABA treated *athb12-3* and *athb12-4* plants produced an *ATHB12* transcript, but at levels that were approximately 10% of the wild-type level (data not shown). In all four *athb12*-mutants, the basal level of *ATHB7* mRNA and the ABA induced increase was similar to that in wild-type (Figure 2B).

The four *athb12*-mutants were similar to wild-type plants in large scale morphology when grown under normal or water deficit conditions (data not shown).

In assays for root growth, the four *athb12*-mutants were indistinguishable from wild-type when grown on medium lacking ABA. On medium supplemented with 10 μM ABA the roots of wild-type seedlings, Col6 and Ws2, grew to approximately 50% of the length of seedlings grown on medium without ABA. Roots of *athb12-1* and *athb12-2* (Figure 2C), and *athb12-3* and *athb12-4* (data not shown) responded like the corresponding wild-types to the presence of ABA in the medium, by a reduction in elongation. The response, however was consistently approximately 10% less than the wild-type response. On media containing ABA at 1, 5, 50 or 100 μM, the *athb12*-mutants showed similar 10% reductions in root sensitivity to ABA (data not shown).

Transgenic plants with high-level expression of ATHB12 *and/or* ATHB7 *show growth patterns similar to those of wild-type plants subjected to water deficit*

To further address the function of *ATHB12*, we transformed wild-type plants with a construct carrying the *ATHB12* cDNA in the sense orientation under the control of the 35S CaMV promoter, to generate five independent homozygous single insert *35S::ATHB12* lines. The same *35S::ATHB12* construct was introduced by transformation into M4 plants (Hjellström *et al.*, 2003) which express ATHB7 at increased level, under the

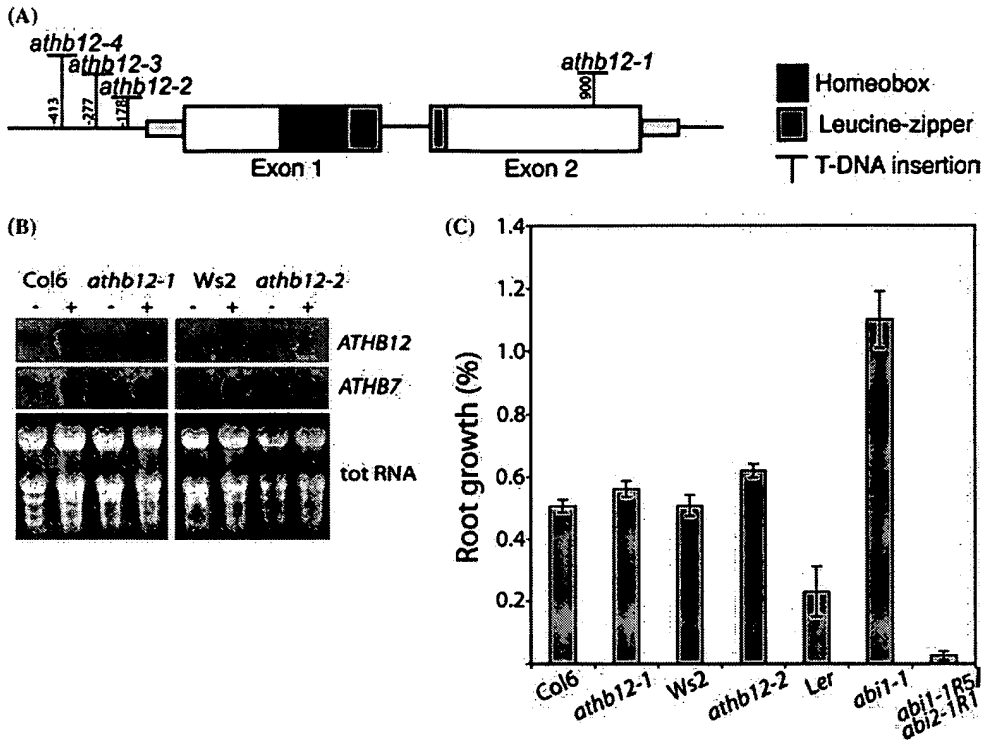
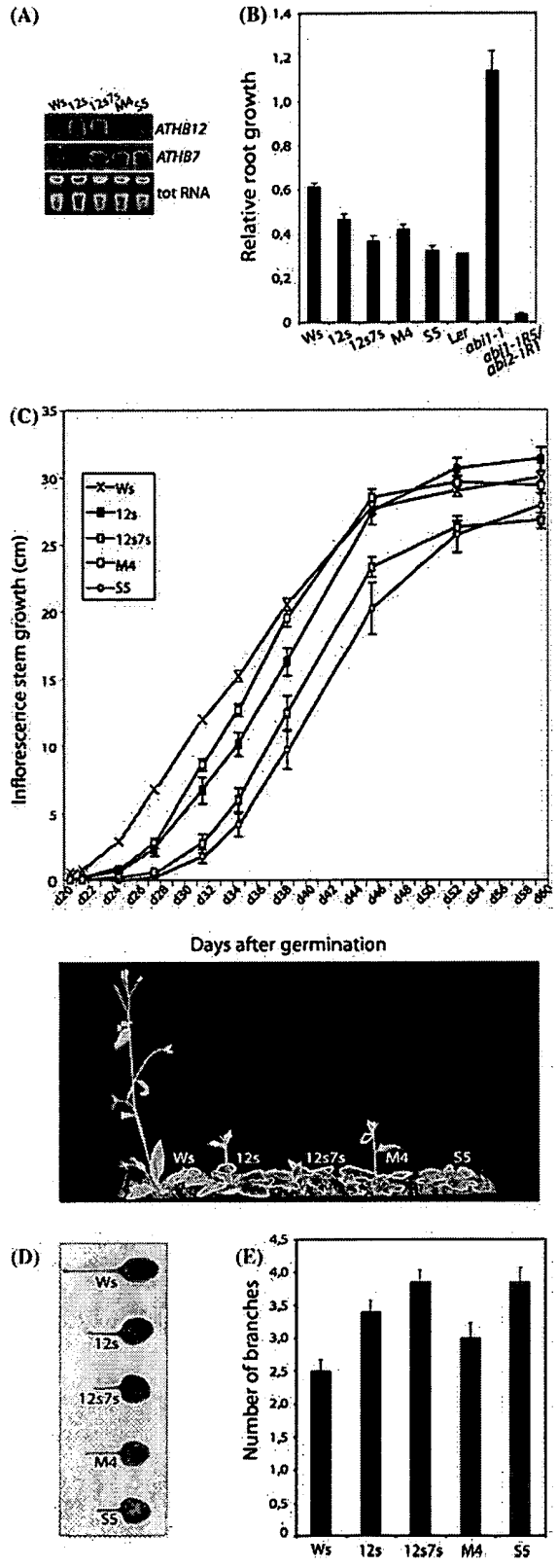


Figure 2. *athb12*-mutant seedlings are insensitive to ABA. (A) A schematic representation of the positions of the T-DNA insertions in the different *athb12*-mutants. (B) Transcript levels of *ATHB12* and *ATHB7* in 10-day-old Col6 (wild-type background to *athb12-1*) *athb12-1*, Ws2 (wild-type background to *athb12-2*) and *athb12-2* seedlings, non-treated (–) or treated with (+) 50 μM ABA for four hours. The bottom panel shows the ethidium bromide stained gel before blotting. (C) Inhibition of root elongation growth of seedlings grown on medium containing 10 μM ABA. The ABA mediated inhibition of root growth over a four days period was calculated as the ratio between the increment in root length of seedlings treated with ABA and untreated control seedlings. The experiment was repeated three times and evaluated with students *t*-test with similar results. One of these experiments is presented. The difference between Col6 and *athb12-1* and between Ws2 and *athb12-2* are statistical significant ($p = 0.04$ and 0.01 respectively, $n = 12$–24). *Ler*, *abi1-1* and *abi1-1R5/abi2-1R1* were included as internal controls ($n < 12$). Error bars represent SE.

control of the 35S-promoter to generate seven independent homozygous single insert transformants *35S::ATHB12;7* lines. The *ATHB12* mRNA levels in the transformant lines ranged from 5 to 30 times the wild-type non-induced level (data not shown). One *35S::ATHB12* line (12s) and one *35S::ATHB12;7* line (12s7s), which both expressed *ATHB12* at a level approximately 26 times the wild-type level under normal growth conditions were selected for further characterisation together with the M4 and one additional *35S::ATHB7* line; S5, which expresses *ATHB7* at a higher level (Figure 3A, Hjellström *et al.*, 2003).

To examine whether high-level expression of *ATHB12* and *ATHB7* affected ABA sensitivity, the germination and root elongation responses to ABA were analysed in the transgenic plants. Root elongation in the transgenic lines did not differ from wild-type in plants grown on medium without ABA (data not shown). The transgenic plants, however, differed from wild-type in their root growth response to ABA. On medium supplemented with 10 μM ABA the inhibition of root growth of both 12s and M4 was enhanced; roots developing to approximately 70% of the length of the wild-type roots (Figure 3B). The response of 12s7s roots to ABA was more strongly enhanced and similar to that of S5. On media containing 1, 5, 50 or 100 μM ABA the 12s and 12s7s plants showed a quantitatively and qualitatively similar enhancement of the ABA-response, as compared to wild-type (data not shown). In assays for germination on ABA supplemented media the transgenic lines did not differ from wild-type (data not shown).

Further, the *35S::ATHB12* and *35S::ATHB-12;7* plants differed from wild-type in the elongation of the inflorescence stem. Compared to wild-



type plants, the inflorescence stem elongation in the different *35S::ATHB12* and *35S::ATHB12;7* plants was delayed by 3–7 days. Among the different *35S::ATHB12* lines, as well as among the *35S::ATHB12;7* lines, the delay in inflorescent stem elongation correlated with the expression levels of *ATHB12* (data not shown). The 12s7s plants showed a retardation of stem elongation that was more severe than that of either the M4 or 12s line (Figure 3C). The S5 line, which expresses *ATHB7* at approximately twice the level of 12s7s, exhibited a retardation in stem elongation similar to that of 12s7s (Figure 3A and C). The time of flower initiation did not differ significantly from wild-type plants in either of the 12s or 12s7s lines (data not shown).

The petioles of the 12s and M4 plants rosette leaves were shorter than those of the wild-type and the 12s7s and S5 plants had even shorter petioles. The leaf blades of the transgenic lines were also more rounded in shape than the wild-type leaves (Figure 3D). The 12s plants, on average, had one more branch on the inflorescence stem than the wild-type. 12s7s and S5 plants had more than one

Figure 3. Characterisation of transgenic plants with constitutive high-level expression of *ATHB12* and/or *ATHB7*. (A) Gene specific probes corresponding to *ATHB12* or *ATHB7* (as indicated in the figure) hybridised to a Northern blot membrane containing RNA samples derived from 10-day-old wild-type seedlings (Ws), or transgenic seedlings expressing *35S::ATHB12* (12s), *35S::ATHB7* (M4) or both the *35S::ATHB12* and the *35S::ATHB7* gene constructs (12s7s), and seedlings expressing a different *35S::ATHB7* construct (S5). The bottom panel shows the ethidium bromide stained gel before blotting. (B) Inhibition of root elongation growth by ABA. Growth of seedlings of different genotypes, for four days on media containing ABA at 10 μM ABA, expressed as a fraction of the growth of control seedlings grown on medium without ABA. The *abi1-1* and *abi1-1R5/abi2-1R1* seedlings were included as ABA insensitive and ABA hypersensitive controls. The difference in ABA response between wild-type and transgenic plants was demonstrated to be of statistical significance (*t*-test, $p < 0.01$). The results are presented as means with error bars representing SE ($n = 16$–24) from four independent experiments. (C) Inflorescence stem elongation in the different genotypes, represented as means ± SE ($n = 7$–12). The lower panel shows a picture of the transgenic lines on day 28 after germination. (D) The fourth rosette leaf from representative individuals of 21-day-old transgenic plants. (E) The mean number of branches on the main inflorescence stem, of 55-day-old plants. Error bars represent SE ($n = 20$). Mann-Whitney-*U* test was performed to verify statistical significance.

additional branch compared to M4 and 12s (Figure 3E).

ATHB12 *and* ATHB7 *promoter activities are similarly regulated by ABA and water deficit*

A 3.2 kb genomic DNA fragment of *ATHB12* containing 2581 nucleotides of the sequence upstream from the transcriptional start site, including the homeobox, the intron and the major part of the sequence corresponding to the leucine zipper, was fused to the reporter gene *uidA*, and the construct was transformed into *Arabidopsis* plants.

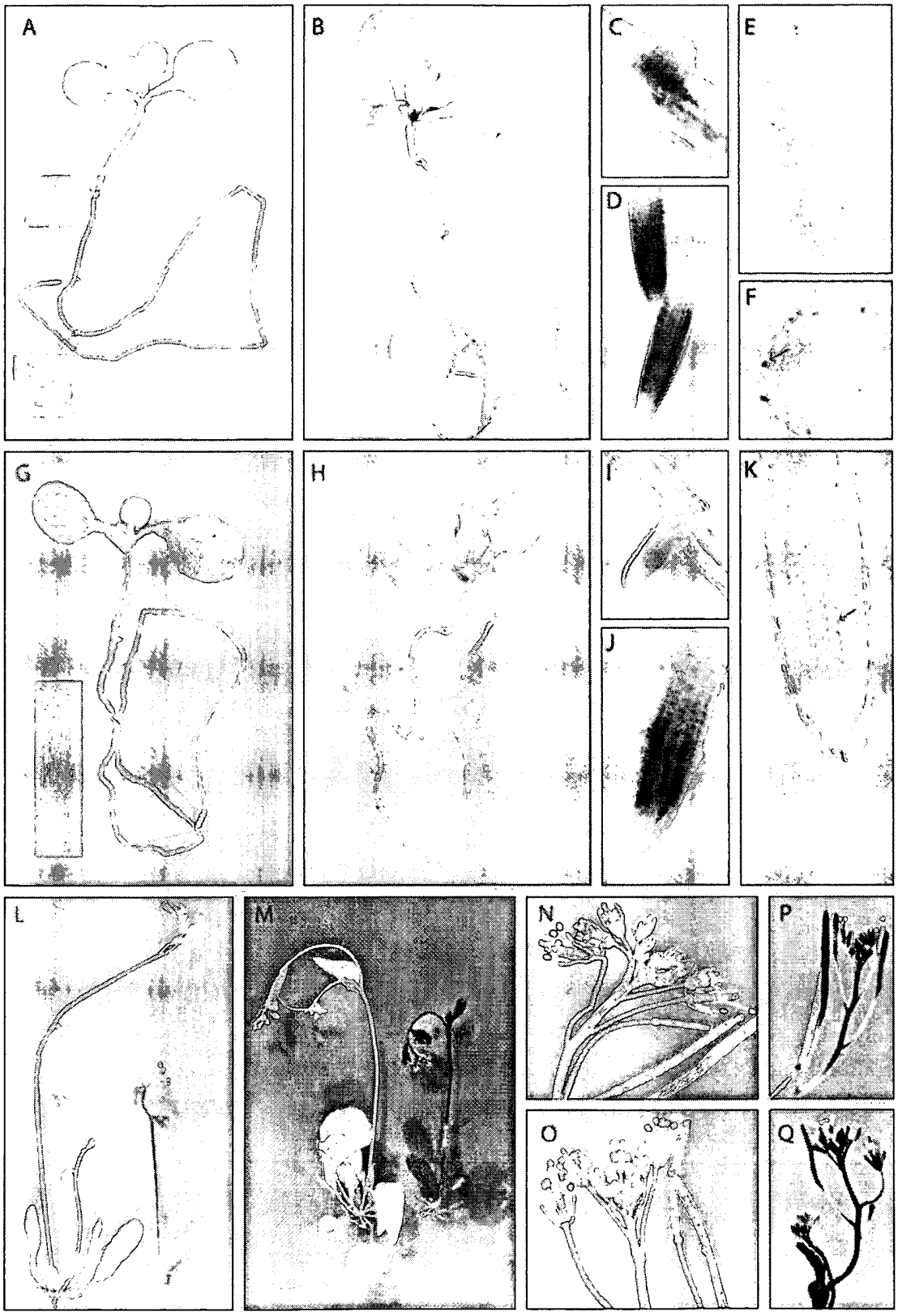
10-day-old *ATHB12::uidA* seedlings grown under normal conditions showed GUS staining at low levels in leaf primordia, developing petioles, the basal part of young rosette leaves and in lateral root primordia (Figure 4A). ABA or water deficit treatments caused the staining in these tissues to increase in intensity and induce *ATHB12* promoter activity in whole leaves, cotyledons, root vasculature and the root tips (Figure 4B, C and D). The staining in the root tip was mainly localised to the cortex and endodermis of the elongation/differentiation zone (Figure 4D, E and F). In root tip sections, strong GUS staining could be detected in a subcellular pattern, which likely reflects a nuclear localisation of the hybrid protein (Figure 4F), consistent with a nuclear function of *ATHB12*.

The *ATHB12::uidA* construct contains eleven putative ABA responsive elements (ABRE; Izawa *et al.*, 1993; Shen and Ho, 1995), and three CE1 elements, two of which are in close proximity to ABREs. The two ABRE-CE1 elements in *ATHB12* reside in a sequence of the promoter that is noticeably similar in sequence to a corresponding *ATHB7* promoter sequence, which also includes two putative ABRE-CE1 elements (Hjellström *et al.*, 2003). *ATHB7::uidA* seedlings (Hjellström *et al.*, 2003) grown under normal conditions showed no detectable GUS activity, except in the root tip, in which two zones of weak gene activity on opposite sides of the differentiation/elongation zone of root tips were observed (Figure 4G). After ABA treatment, GUS staining in this region increased in intensity and extended to surround the whole root tip in all cell layers, and showed a subcellular distribution similar to that of the ATHB12-GUS protein (Figure 4H, I, J and K). GUS staining was also detected in the root vasculature, lateral root primordia, leaf primordia, cotyledons and in young leaves (Figure 4H and I).

Promoter activities of *ATHB12* and *ATHB7* in adult plants grown under well-watered conditions were high in axillary buds (Figure 4L; Hjellström *et al.*, 2003) and *ATHB12* activity was also present in the vasculature of leaves and in inflorescence stems (Figure 4L and N). In the flowers, *ATHB12::uidA* expression could be detected (Figure 4N) in the expanding gynoecia of young flower buds in stage 5–12 (Smyth *et al.*, 1990). After pollination, at stage 14, GUS staining was observed in basal part of anther filaments and below stigmatic papillae. At stage 17A (Ferrandiz *et al.*, 1999), when all organs had abscised from the receptacle, the nectaries stained blue and the *ATHB12* promoter was also active in developing seeds (Figure 4N). The *ATHB7::uidA* plants grown parallel to these plants showed similar staining in anther filaments and in developing seeds (Figure 4O; Hjellström *et al.*, 2003).

Adult plants responding to water deficit, as indicated by decreased growth of the inflorescence stem and leaves and a tendency to wilt, showed *ATHB12::uidA* activity in the inflorescence stem, the cauline- and rosette leaves, at high levels in the vasculature (Figure 4L and P). GUS activity was also observed in all organs of developing flowers and elongating siliques, particular their receptacles (Figure 4P). Adult *ATHB7::uidA* plants grown under the same conditions showed high gene activity in the inflorescence stem, the rosette leaves; particularly in the vasculature, and in flower organs (Figure 4M and Q; Hjellström *et al.*, 2003).

In the water deficit experiments on adult plants, described above, *ATHB12::uidA* and *ATHB7::uidA* plants were harvested in parallel for RNA-extraction from the rosette leaves and the apical and the basal parts of the inflorescence stems. RNA gel blot analyses were performed and *ATHB12*, *ATHB7* and *uidA* gene specific probes were hybridised to the blotted membranes. The results showed that the ratio of the levels of *ATHB12* or *ATHB7* transcript to that of each *uidA*-transcript was similar in the different samples (data not shown), indicating that the GUS staining patterns represented *ATHB12* and *ATHB7* expression in the different plants.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Discussion

In this report we present an analysis of the regulation and the function of the homeodomain-leucine zipper (HDZip) gene *ATHB12* in *Arabidopsis*. We demonstrate that the expression of the gene is dependent on water availability to the plant, and on ABA signalling. In these aspects of the gene activity, *ATHB12* is similar to the paralogous gene, *ATHB7* (Söderman, *et al.*, 1996). The *ATHB12* and *ATHB7* share similarities in sequence, a common intron–exon organisation and similar specificities in DNA-binding (Johannesson *et al.*, 2001), that distinguish the two paralogs from other class I HDZip genes in *Arabidopsis*.

Our data on the transcriptional regulation of *ATHB12* and *ATHB7* in different ABA synthesis and signalling mutants demonstrate that the two genes are highly similar as regards the mechanisms by which their expression is controlled by the water status of the plant and to abscisic acid signalling and, thus, indicate that both genes may have regulatory roles related to ABA signalling and act in the plant response to water limiting conditions. The specific roles of *ATHB12* and *ATHB7* in the ABA signalling mechanism are difficult to deduce from our data on the *abi1* and *abi2* mutants, since the mechanisms of actions in the ABA response of ABI1 and ABI2 are poorly understood. Our finding that *ATHB12* and *ATHB7* induction by ABA is impaired in the dominant *abi1-1* and *abi2-1* mutants, as well as in plants carrying the presumed loss-of-function revertant alleles of the genes (*abi1-1R4*, *abi1-1R5* and *abi2-1R1*) is in apparent contradiction to the reported phenotypes of these mutants. Previous data (Gosti *et al.*, 1999; Merlot *et al.*, 2001) has demonstrated the revertant plants to have an enhanced sensitivity to ABA, whereas the *abi1-1* and *abi2-1* mutants are insensitive to ABA. However, both categories of mutants are reported to have reduced phosphatase activity, as a result of the mutations (Bertauche *et al.*, 1996; Leung *et al.*, 1997; Gosti *et al.*, 1999; Merlot *et al.*, 2001). Therefore, it is possible that the reduced response of *ATHB12* and *ATHB7* in both classes of mutant reflects a requirement for ABI phosphatase activity as part of the mechanism that leads to the transcriptional activation of *ATHB12* and *ATHB7*.

Our data also supports the notion proposed by Merlot *et al.* (2001) that ABI1 and ABI2 are functionally redundant, since the *abi1/abi2* double loss-of-function mutants show a more severe deviation from wild-type than either of the single mutants in their transcriptional response of *ATHB12* and *ATHB7* to ABA. The residual, low level response of *ATHB12* and *ATHB7* to ABA in the double mutants may be due to the activities of additional redundant PP2Cs, possibly AtPP2CA and HAB1, which like ABI1 and ABI2 are suggested to negatively regulate ABA signalling (Rodriguez *et al.*, 1998; Sheen, 1998; Tähtiharju and Palva, 2001; Saez *et al.*, 2004).

In addition to the similarities in the mechanism by which the *ATHB12* and *ATHB7* are up-regulated in response to water limiting conditions, the genes also share extensive similarities in their spatial patterns of expression in the plants. Both genes are expressed in a wide range of organs, primarily in ontogenetially young and expanding tissues, and their expression domains essentially overlap. Therefore, *ATHB12* and *ATHB7*, at least to a large extent, may have overlapping functions in a range of different organs.

Our functional data from transgenic *Arabidopsis* plants which express *ATHB12* and/or *ATHB7* at high-levels, support this notion, in that

Figure 4. Histochemical localisation of GUS activity in *ATHB-12::uidA* and *ATHB7::uidA* plants. GUS activity in 10-day-old *ATHB12::uidA* seedlings grown under normal conditions [(A), inserted are enlarged sections showing staining in lateral root primordia], or treated with 50 μM ABA (B, C, D, E and F). Figure E shows a longitudinal section and F a transverse section of a root tip, the arrow in F indicates staining in a part of the cell that likely corresponds to the nucleus. Figure (G–K) shows *ATHB7::uidA* seedlings, in G grown under normal conditions (insert shows an enlargement of a root tip) and in H, I, J and K plants of the same genotype treated with 50μM ABA. Figure K shows a longitudinal section of a root tip and the arrow in K indicates staining of a cell compartment that likely corresponds to the nucleus. Figure L and M show 28-day-old plants grown on well-watered soil (left plants in L and M, inflorescence in N and O) or under water deficit conditions, as a result of withholding water for approximately two weeks (right plant in L and M and inflorescence in P and Q). L, N and P show *ATHB12::uidA* plants (arrow pointing to an axillary shoot bud in L) and M, O and Q *ATHB7::uidA* plants. Both control and plants exposed to water deficit conditions were submerged and sectioned longitudinally in their stems in GUS staining solution to allow efficient penetration of the staining solution. The unstained part of the stem of the water deficit exposed *ATHB7::uidA* plant (indicated by an arrow in M) was not sectioned, as a control.

the plants show similar phenotypic deviations from wild-type in inflorescence stems and leaves as well as in the root. For both genes the phenotypic effects are restricted to the post-germinative phase of growth. *Arabidopsis* responds to water deficit conditions by reduced growth of the inflorescence stems, leaves and roots (Himmelbach *et al.*, 1998). This growth pattern is essentially mimicked by the phenotypes of *ATHB12* and/or *ATHB7* expressing transgenic plants. The phenotypic deviation in the above ground parts of the plants differed from the root-phenotype in that the effect of high-level expression of *ATHB12* and *ATHB7* on root development was apparent only after ABA treatment i.e., conditional on water-stress signalling. Shoot growth is very sensitive to inhibition by water limiting conditions whereas root growth is usually less affected (Saab *et al.*, 1990). The mechanisms responsible for this difference in sensitivity between root and shoot growth in response to water stress are not well understood, but ABA, sugar and ethylene signalling have been implicated. The involvement of *ATHB12* and *ATHB7* in the growth response to water deficit in the shoot as well as the root suggests that the drought response in both organs is regulated by common signalling mechanisms, which include *ATHB12* and *ATHB7*.

In support of the notion that *ATHB12* and *ATHB7* act directly as negative regulators of stem elongation, the level of *ATHB12*/*ATHB7* transcript in the transgenic over-expressor lines correlated quantitatively with the reduction in stem elongation. Further, plants that express both genes at high-levels showed more severe phenotypes than plants expressing each single gene at elevated levels. The phenotypic properties of the 12s7s plants were similar to those of the S5 line, which expressed *ATHB7* at a level approximately twice that of 12s7s but showed wild-type levels of *ATHB12* expression. These data indicate that the effects of increases in expression levels of *ATHB12* and *ATHB7* are additive, and that the two genes either have similar targets or that their targets, if different, have similar effects on plant growth and that these effects are additive.

The absence of a mutant phenotype in the above ground parts of the *athb12*-mutant presented in this report may be due to the capacity of *ATHB7* to substitute functionally for the loss of *ATHB12* in these mutants. We note that this potential functional redundancy between the genes is not complete, since the *athb12*-mutants differ from wild-type in the root response to ABA, indicating that *ATHB12* is essential for this response, and that *ATHB7* cannot substitute for *ATHB12* in this aspect of the ABA response.

The functions of *ATHB12* and *ATHB7* as regulators of cell elongation is interesting in relation to the roles of other class I HDZip proteins, which have also been implicated in the regulation of cell expansion in response to different environmental stimuli. *ATHB13*, which has a function related to sucrose signalling (Hanson, 2000), and the closely related *ATHB3*, *ATHB20* and *ATHB23* are suggested to act as negative regulators of lateral cell expansion in leaves (Mattsson *et al.*, 1992; Hanson *et al.*, 2001). *ATHB16* has a role in the regulation of leaf cell expansion, but in response to light (Wang *et al.*, 2003). The related *ATHB5* and *ATHB6* genes are thought to mediate ABA responses (Himmelbach *et al.*, 2002; Johannesson *et al.*, 2003), and *ATHB5* is suggested to be a negative regulator of cell expansion (Johannesson *et al.*, 2003). Even though homodimers of ATHB12 or ATHB7 do not interact *in vitro* with the target sequence recognised by other HDZip factors, ATHB12 and ATHB7, as well as ATHB6 and ATHB16 can form heterodimers *in vitro* with ATHB5 (Johannesson *et al.*, 2001). This implies that the class I HDZip proteins may constitute a network of interacting factors that mediate responses to environmental stimuli of different kinds, and integrates information on environmental conditions to regulate similar sets of target genes.

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Constitutive expression of the water deficit-inducible homeobox gene *ATHB7* in transgenic *Arabidopsis* causes a suppression of stem elongation growth

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ABSTRACT

The *Arabidopsis* gene *ATHB7* encodes a transcription factor of the homeodomain-leucine zipper class. The activity of the gene is dependent on the water conditions of the plant; expression being strongly induced in plants subjected to water deficit or to treatment with abscisic acid (ABA). In this report we demonstrate that *ATHB7*, when constitutively expressed at levels typical for plants exposed to water-deficit conditions, caused a reduction in elongation growth in the leaf and in the inflorescence stem. The reduction in stem growth mimics the effect on growth caused by water-deficit conditions, but is independent of water conditions in plants that express *ATHB7* constitutively. These results indicate that ATHB7 in the wild-type plant may be a mediator of the plant growth response to limiting water conditions in the leaf and in the inflorescence stem. In support of this notion we also show that the *ATHB7* promoter in wild-type plants exposed to water-deficit conditions is highly active in the elongating parts of the inflorescence stem as well as leaves.

Key-words: *Arabidopsis thaliana*; abscisic acid; abscisic acid responsive elements; homeodomain; leucine zipper; transcription factor; water deficit.

INTRODUCTION

Plants exposed to different environmental conditions, such as different light- and water-availability and temperature, adapt by altering their growth characteristics. In response to water-deficit conditions several morphological, physiological and biochemical processes are altered. The immediate response includes the closure of stomata in leaves, which reduces further water loss, a process mediated by the plant hormone abscisic acid (ABA) (for reviews, see Finkelstein & Zeevaart 1994; Leung & Giraudat 1998). Morphological changes due to extended water deficit include an inhibition of stem elongation, a reduction in leaf area and an increase in root growth (Trewavas & Jones 1991). Cellular responses to water-limiting conditions include modifications of the lipid composition of the cytoplasmic membrane and synthesis of osmoprotectant solutes (for reviews see Bray 1993; Daugherty *et al.* 1994).

A common factor mediating most but not all plant responses to water deficit, high salinity and cold is ABA. It has been shown that the endogenous level of ABA increases in response to drought and cold (Chen, Li & Brenner 1983; Lalk & Dörffling 1985; Lång *et al.* 1994) and that treatment with ABA makes many plants more resistant to drought (Bartels *et al.* 1990), salt (LaRosa *et al.* 1987) and cold (Chen & Gusta 1983). Using the ABA-deficient *aba* mutant (Koornneef *et al.* 1982) it has further been demonstrated that ABA is required for stomatal closure as well as for the development of freezing tolerance in *Arabidopsis* (Heino *et al.* 1990). Consequently, the elucidation of the mechanisms involved in the control of gene expression in response to ABA is important for understanding the mechanisms which control adaptive processes in response to water deficit. Recently, substantial progress has been made in the characterization of ABA signal transduction cascades (Bonetta & McCourt 1998; Busk & Pagès 1998). ABA signalling seems to involve a complex network (Söderman *et al.* 2000) of kinases and phosphatases with both positive- and negative-regulating activities (Gosti *et al.* 1999; Merlot *et al.* 2001).

Long-term changes in plants in response to water deficit, high salinity and cold are linked to changes in gene expression. Specific genes are transcriptionally activated, several of them also by exogenous treatment with ABA (Skriver & Mundy 1990; Chandler & Robertson 1994). The genes differ in the specificity of their response to drought, cold and ABA. *Cis*-acting elements and *trans*-acting factors involved in the ABA response have been identified. The most well-characterized class of *cis*-acting elements among the ABA-responsive gene promoters are the abscisic acid responsive elements (ABREs). One example is the Em promoter from wheat (Marcotte, Russell & Quatrano 1989) where a region that is important for ABA responsiveness, which also interacts with nuclear proteins, has been identified (Guiltinan, Marcotte & Quatrano 1990). The transcription factor,

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EmBP-1, which binds specifically to this sequence element, belongs to the bZip class of transcription factors.

We have previously reported on the isolation and characterization of the *Arabidopsis* homeodomain-leucine zipper (HD-Zip) transcription factor gene *ATHB7* (Söderman *et al.* 1994), which is induced by water-deficit conditions as well as by ABA (Söderman, Mattsson & Engström 1996). This class of transcription factor also includes *ATHB6* and *ATHB12* (Lee & Chun 1998; Söderman *et al.* 1999), two genes that are also up-regulated by water-deficit conditions and by ABA. With the recent sequencing of the *Arabidopsis* genome the total number HD-Zip proteins in *Arabidopsis* has been determined to be 42 (Arabidopsis Genome Initiative, AGI 2000). Based on sequence criteria, the HD-Zip proteins have been grouped into four classes, HD-Zip class I–IV (Sessa *et al.* 1994). *ATHB6, -7*and *-12* all belong to HD-Zip class I. Other class I proteins in *Arabidopsis* include for example ATHB13, which has been suggested to function in sucrose signalling (Hanson, Johannesson & Engström 2001). Experiments on transgenic plants and expression analyses in *Arabidopsis* of the HD-Zip class I gene *ATHB1* (Aoyama *et al.* 1995), the class II gene *ATHB2* (Carabelli *et al.* 1996; Steindler *et al.* 1999), the class III genes *ATHB8* (Baima *et al.* 1995) and *IFL1* (Zhong & Ye. 1999) and the class IV genes *ATHB10* (Rerie, Feldmann & Marks 1994; DiCristina *et al.* 1996; Masucci *et al.* 1996) and *ATML1* (Lu *et al.* 1996) suggests that the HD-Zip genes might control important aspects of plant development.

In a previous study we suggested *ATHB7* to act in ABA signal transduction as a mediator of a water-deficit response in *Arabidopsis*, downstream to the *ABI1* gene (Söderman *et al.* 1996), which encodes a serine/threonine phosphatase (Leung *et al.* 1994; Meyer, Leube & Grill 1994). In this study we show that the *ATHB7* promoter is active in the elongating parts of the inflorescence stem as well as in young leaf primordia. We have also investigated the role of ATHB7 by use of transgenic *Arabidopsis* plants with altered levels of expression of *ATHB7*. Together, these data indicate that the role of ATHB7 may be to regulate inflorescence stem and leaf elongation growth in response to water-deficit stress conditions.

MATERIALS AND METHODS

Library screening and DNA sequencing

A λEMBL-3 genomic library containing partially digested *MboI* fragments of *Arabidopsis thaliana* Columbia DNA was screened using an 845 bp *Xho*I – *Eco*RI fragment of *ATHB7*, not containing the conserved homeobox, as a probe. The probe was labelled with [α-^{32}P] dCTP (3000 Ci mmol^{-1}; Amersham Biosciences, Uppsala, Sweden) using the Megaprime DNA labelling kit (Amersham). About 100 000 clones were plated on each of two 23 cm × 23 cm plates and replica filters (Hybond-N, Amersham) were lifted. The filters were hybridized as recommended by the filter supplier and washed at moderate stringency [1 × SSPE (Sambrook, Fritsch & Maniatis 1989); 0.1% sodium dodecyl sulphate (SDS)] at 65 °C for 3 × 15 min. X-ray films were exposed to the filters between intensifying screens at –70 °C.

The λ-DNA was prepared from a single isolated positive plaque, digested with different restriction enzymes, subjected to gel electrophoresis and blotted onto filters (Hybond-N) according to standard methods (Sambrook *et al.* 1989). The filters were hybridized to a probe containing a 516 bp *Hin*dIII-*Acc*I fragment from *ATHB7* cDNA. Overlapping fragments from a 8 kb *Eco*RI fragment, a 3 kb *Sal*I fragment and a 3 kb *Hin*dIII fragment was subcloned into pBluescript SK + (Stratagene, La Jolla, CA, USA).

Sequencing of the 3 kb *Sal*I fragment and the 3 kb *Hin*dIII fragment were performed using the chain-termination method (Sanger, Niklen & Coulson 1977) with Sequenase DNA polymerase (USB, Cleveland, OH, USA) or by use of cycle sequencing, using synthetic oligonucleotides and an automatic sequencer, ABI PRISM 377 (Perkin-Elmer Corp., Applied Biosystems Division, Foster City, CA, USA).

Map position of ATHB7

The chromosomal location of *ATHB7* was determined by use of recombinant inbred (RI) lines (Lister & Dean 1993). Segregation analysis of *Eco*RV restriction fragment length polymorphism among 65 RI lines was performed according to Reiter, Young & Scolnik (1992), using the 8 kb *Eco*RI genomic fragment as a probe. The mapping was carried out using the program MAPMAKER (Lander *et al.* 1987).

Construction of an ATHB7 promoter fusion and plant transformation

A 2.9 kb *Hin*dIII-*Xho*I DNA fragment of the *ATHB7* genomic sequence containing 2.6 kb of upstream sequences was subcloned in frame with the GUS coding sequence (*uidA*; Jefferson, Kavanagh & Bevan 1987) in the pBI101 binary vector (Clontech, Palo Alto, CA. USA). The construct was checked by sequencing and introduced into the MP90 *Agrobacterium tumefaciens* strain by standard methods. The resulting *Agrobacterium* strain was used to transform *Arabidopsis thaliana* ecotypes Wassilevskija, Landsberg and Columbia by an infiltration protocol (Bechtold, Ellis & Pelletier 1993), and transformants were selected on 50 μg mL^{-1} kanamycin. The resultant transgenic plants were self-fertilized and the T_2 seeds screened for 3 : 1 segregation on kanamycin. Homozygous T_3 seeds from three independent transformant plants were used for further characterization.

Plant material and growth conditions

The plant material used was *Arabidopsis thaliana* (L.) Heynh., ecotypes Columbia, Wassilevskija (Ws-0) and Landsberg erecta (*Ler*) and plants homozygous for the *ga1-*

5 allele. In the experiments involving the *ga1-5* mutant, which is in the Landsberg *erecta* (*Ler*) ecotype background, the *Ler* ecotype was used as a wild-type control.

Seeds were surface sterilized (70% EtOH for 2 min, 15% chlorine, 0.5% SDS for 10 min followed by three washes in sterile water) and sown on GM medium [0.5 × MS (Murashige and Skoog 1962); Duchefa Biochemie B.V., Haarlem, The Netherlands] supplemented with 1% sucrose. After 12 d of growth on solid GM medium the plants were put on soil mixed with vermiculite (3 : 1). Prior to cultivation, seed dormancy was broken by 3 d of cold treatment (4 °C). All plants were cultivated in controlled environmental chambers at 20–22 °C, soil-grown material under long day conditions (16 h light : 8 h darkness) and *in vitro*-grown plants in 12 h light : 12 h darkness. Light intensity generated by the fluorescent lights was 200 μE m^{-2} s^{-1}. Water-deficit stress treatment of the transgenic plants, carrying the promoter GUS construct, was performed by withholding water from the plants until they showed a visible water-deficit response, approximately 3 to 6 d prior to analysis. Water-deficit stress treatments of the *35S::ATHB7* transformant plants, starting at initiation of reproductive development, was performed by withholding water to the degree that the water-stressed plants were visibly but not permanently wilting.

Histochemical localization of GUS activity

Histochemical staining for GUS activity was performed using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) as chromogenic substrate (Jefferson *et al.* 1987). Plant material was cut and incubated in GUS staining solution containing 50 mM sodium phosphate pH 7.0; 0.1% Triton X-100; K3/K4 FeCN 0.5 mM and 1 mM X-gluc at 37 °C for 12–24 h. Tissues were cleared of chlorophyll in 70% ethanol (see Fig. 2a & b) or according to the method of Berleth & Jürgens (1993) except that the incubation in chloralhydrate was exchanged for a 16 h incubation in 8 N NaOH followed by 1 h in 0.1 M potassium phosphate buffer, pH 7.5 (see Fig. 2c & d). Photographs were taken using a stereomicroscope.

Generation of transgenic plants with *ATHB7* sense and antisense constructs

The full length *ATHB7* cDNA (Söderman *et al.* 1994) was cloned in sense or antisense orientation downstream of the 35S promoter in the expression vectors pHTT 202 (Elomaa *et al.* 1993) and pBin-HYG-TX (Gatz, Kaiser & Wendenburg 1991) and introduced into the *Agrobacterium tumefaciens* strain C58::pGV2260 by triparental mating. The resulting *Agrobacterium* strain was used to transform *Arabidopsis thaliana* ecotype Wassilevskija and Landsberg *erecta* by an infiltration protocol (Bechtold *et al.* 1993), and transformants were selected on 50 μg mL^{-1} kanamycin (pHTT202) or hygromycin (pBin-HYG-TX). The resultant transgenic plants were selfed and the T_2 seeds screened for 3 : 1 segregation. Homozygous T_3 seedlings were analysed for *ATHB7* RNA levels by Northern blot hybridization and used for further characterization.

RNA isolation and Northern blot analysis

Total RNA was isolated according to Söderman *et al.* (1996). Samples of total RNA, 10 μg per lane, were subjected to electrophoresis in a 1% agarose gel containing formaldehyde and blotted onto nylon membrane (Hybond-N; Amersham International, Buckinghamshire, UK). Equal loading was confirmed by ethidium bromide staining of the agarose gel. The filters were hybridized to the 516 bp *Hin*dIII-*Acc*I fragment from *ATHB7* cDNA not containing the conserved homeobox. The probe was labelled with [α-^{32}P] dCTP (3000 Ci $mmol^{-1}$, Amersham) using the Megaprime DNA Labelling Kit (Amersham). Prehybridization and hybridization was performed at 63 °C as described by the filter supplier and the filters were washed at high stringency (0.1 × SSPE; 0.1%SDS) at 65 °C for 2 × 5 min. Quantitative data on hybridization were obtained by use of a BAS 2000 (Fuji Photo Film Co., Tokyo, Japan) image plate reader.

Rosette leaf area measurement

Rosette leaves were collected from six wild-type and from six *35S::ATHB7* transformant plants at 24–32 d after germination. The areas of individual rosette leaves were measured using NIH Image 1.61 (National Institutes of Health; http://rsb.info.nih.gov./nih-image/) and the total area of the rosette leaves of each plant was calculated.

Hormone treatments

To test stem elongation responses, plants were sprayed with 1 mM GA3 (Duchefa, Haarlem, The Netherlands) and 0.02% Tween-20 approximately 1 week before bolting. The plants were then sprayed every fourth or fifth day until harvest. The control plants were sprayed with a solution containing only 0.02% Tween-20.

Scanning electron microscopy

Plants were harvested at an inflorescence stem length of 100 mm, and 10 mm fragments from the central parts of the first internode were sampled. Tissues were fixed in 50% EtOH, 5.0% acetic acid and 3.7% formaldehyde and exposed to 30 min vacuum. After dehydration through an ethanol series (50% EtOH for 2 × 30 min, 60% EtOH for 30 min and 70% EtOH until no chlorophyll remained), samples were stored in 70% ethanol until exposure to 85% and 95% EtOH and critical point drying. Samples were coated with gold, analysed in a XL 30 scanning electron microscope (SEM; Philips Technologies, Cheshire, CT, USA) and micrographs were taken. Six samples from each plant line were analysed, and from each sample at least four cells were measured with respect to length Using NIH Image 1.61 (National Institutes of Health).

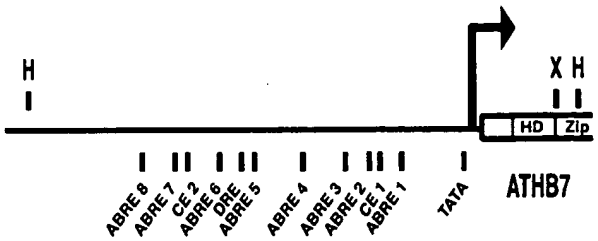
RESULTS

Isolation of a genomic clone corresponding to ATHB7

By use of a probe derived from the 3′-end of the *ATHB7* cDNA (Söderman *et al.* 1994), three overlapping clones were isolated from a genomic *Arabidopsis* library. A 3.0 kb *Hin*dIII clone was sequenced and shown to contain upstream sequences and 350 bp of the sequence corresponding to the *ATHB7* cDNA. This clone was further characterized (Fig. 1).

The map position of the *ATHB7* locus was determined to the bottom of chromosome 2 by use of recombinant inbred (RI) lines (Lister & Dean 1993). This position is consistent with the sequencing data derived from the *Arabidopsis* genome project (AGI 2000). As deduced from a comparison between the cDNA and the genomic sequence, the *ATHB7* gene contains only one intron. This intron is located within the leucine zipper region in a position conserved among class I HD-Zip proteins. Data from the rapid amplification of 5′-cDNA ends revealed the untranslated leader to be 60 bp long. A sequence identical to the consensus sequence of transcriptional initiation sites (CTCATCA) (Joshi 1987) is located at a position 70 bp upstream from the translation initiation site of the longest open reading frame. Further, a putative TATA box (TATATAA) is found at a position 22 nucleotides upstream from this site.

The 5′ upstream promoter region of the *ATHB7* gene includes eight different sequence motifs similar to the consensus sequence of the ABRE (Fig. 1). Two of the ABREs, at position −762 and −2052, respectively, were accompanied by putative coupling elements (CACC) (Shen & Ho 1995; Shen, Zhang & Ho 1996). Sequence analysis also revealed a putative drought-responsive element (DRE), with the core sequence CCGAC, at position −1674 from the transcriptional start site.



Element	Position	Sequence	Element	Position	Sequence
TATA	-71	TATATAA	ABRE 5	-1662	AACGTA
ABRE 1	-723	AACGTT	DRE	-1674	TTCCGACAA
CE 1	-750	CACC	ABRE 6	-1870	TACGTT
ABRE 2	-762	TACGTA	CE 2	-2020	CACC
ABRE 3	-1008	TACGTG	ABRE 7	-2052	CACGTG
ABRE 4	-1322	AACGTT	ABRE 8	-2278	AACGTG

Figure 1. Genomic organization of *ATHB7*. Schematic representation of promoter and upstream sequences of the *ATHB7* gene. The location of the encoding region in the 3′- end of the genomic clone is indicated by a box. Positions of the homeodomain and the leucine zipper are indicated within the box. The location of the transcriptional start site is indicated with an arrow. The position of motifs similar to a TATA box, the consensus sequence of abscisic acid responsive elements (ABRE), putative coupling elements (CACC) and a drought-response element (DRE) are indicated. *Hin*dIII and *Xho* I endonuclease restriction sites are indicated with H and X, respectively.

ATHB7::GUS is expressed in expanding organs of transgenic *Arabidopsis* plants exposed to water-deficit stress

In order to characterize the cellular and tissue distribution of *ATHB7* promoter activity, we analysed the expression of an *ATHB7*::GUS chimeric gene in transgenic *Arabidopsis* plants. A 2.9 kb genomic *Hin*dIII-*Xho*I fragment (Fig. 1) of *ATHB7* including 2.6 kb of upstream and promoter region was cloned as a translational fusion in frame with the coding sequence of the GUS reporter gene (*ATHB7*::GUS) and transgenic *Arabidopsis* plants were generated with this construct. Three independent *ATHB7*::GUS transformant lines showing a 3 : 1 segregation of the resistance marker in the T_2 generation were selected for further analysis.

Transgenic *ATHB7*::GUS plants, histochemically stained for GUS activity are shown in Fig. 2. Plants grown under optimal water conditions showed no or very low GUS activity in all organs and tissues analysed, with an exception for the axillary buds that showed strong GUS activity (Fig. 2a). In contrast, the GUS activity was strongly induced in plants exposed to limiting water supply. In adult plants, high promoter activity could be detected in the developing leaf primordia and in inflorescence stems and flowers of plants exposed to extended water limitation (Fig. 2b & d). The GUS activity was most intense in the young parts of the inflorescence and the elongating parts of the stem. Water deficit-induced promoter activity was also detected in the expanding siliques (Fig. 2d). In plants exposed to limiting water supply, strong GUS activity was detected in young leaves but at later stages of leaf development, expression was maintained at a moderate level only in the vascular tissue (data not shown). In well-watered plants, the axillary bud (Fig. 2a) showed the most intense GUS staining in the developing leaf primordia and low or no expression in the meristematic tissue (data not shown).

Phenotypic effects of alterations in *ATHB7* expression levels in transgenic *Arabidopsis*

Transgenic *Arabidopsis* plants harboring the *ATHB7* cDNA in the sense or antisense orientations under the control of the 35S CaMV promoter were generated in the Wassilevskija (WS) background. Ten independent sense (*35S::ATHB7*) and five independent antisense transformant lines (*35S::αATHB7*) that showed a 3 : 1 segregation of the resistance marker in the T_2 generation were selected for further analysis.

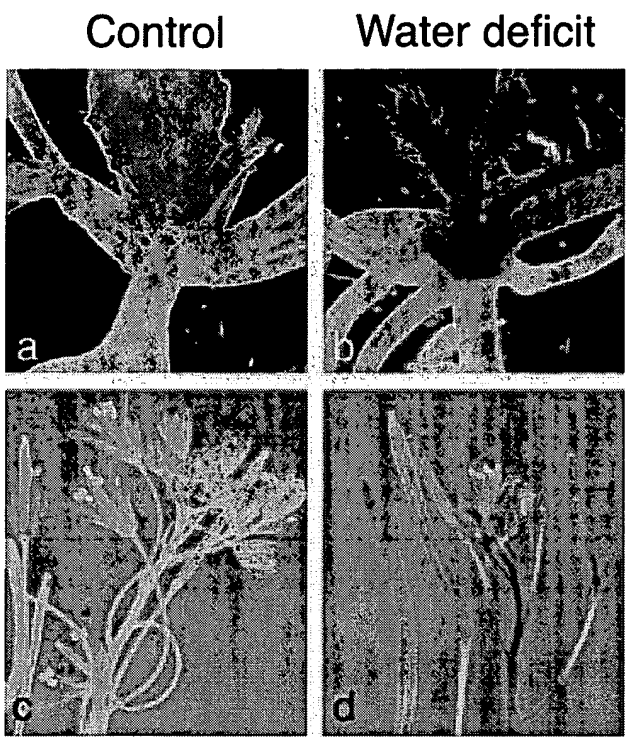


Figure 2. *ATHB7*::GUS gene expression in transgenic *Arabidopsis* plants. Histochemical localization of GUS activity in (a); shoot of a 23-day-old plant grown in non-limiting water conditions (b); shoot of 23-day-old plant subjected to limiting water conditions (c); inflorescence of an adult plant grown in non-limiting water conditions (d); inflorescence of an adult plant subjected to limiting water conditions.

Among the five antisense lines, two lines showed 30 and 60% reduction in transcript levels, respectively, when analysed in a Northern blot experiment. None of these lines showed any apparent difference from wild type, when grown under optimal or limiting water conditions.

The 10 *35S::ATHB7* lines were analysed for *ATHB7* mRNA expression levels by Northern blot analysis. The transcript levels ranged from two- to 25-fold the wild-type level. For comparison, *ATHB7* expression levels in wild-type plants subjected to 10 μM ABA was determined to eight times the wild-type control after 4 h of ABA treatment. Two lines, S5 and M4, with elevated levels of *ATHB7* transcription were selected for further analysis (Fig. 3a). The S5 line had a 25-fold increase in *ATHB7* expression compared to wild type and the M4 line a 20-fold increase in *ATHB7* expression. The most striking phenotypic alterations in these lines were a suppression of inflorescence stem elongation growth and increased branching of the main inflorescence stem (Fig. 3b–f). The occurrence of this

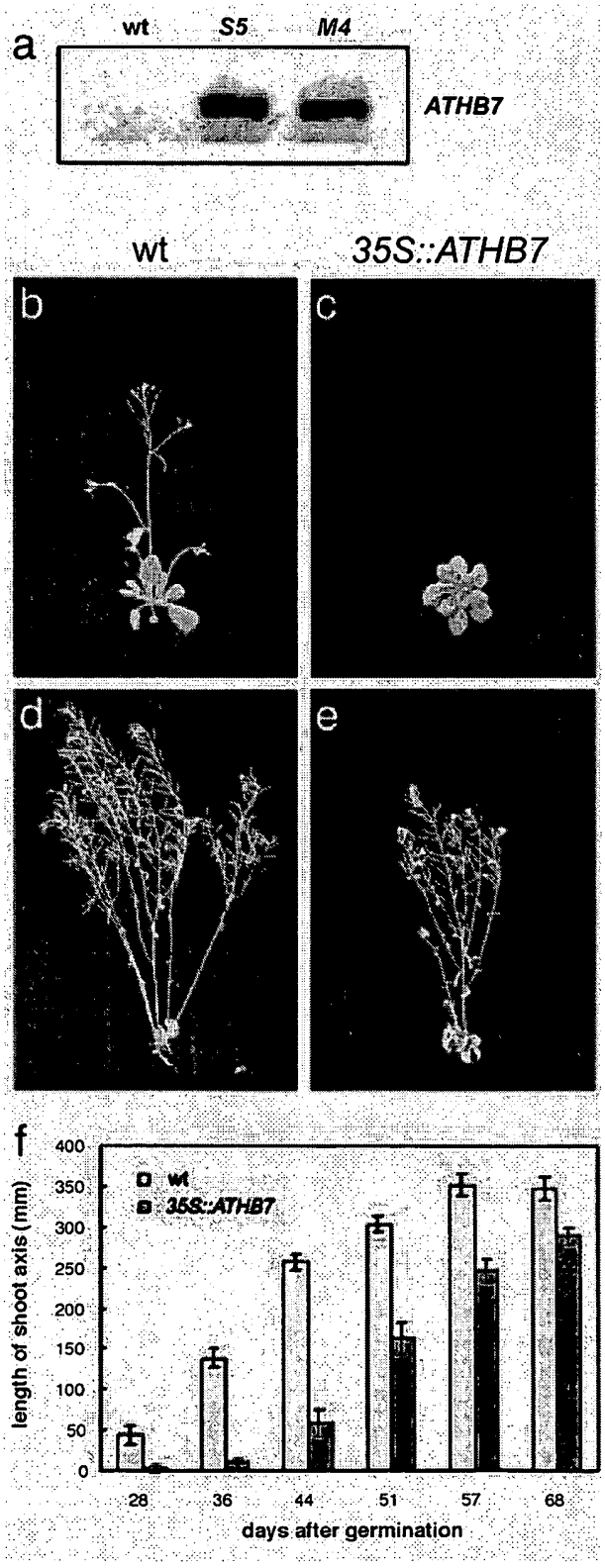


Figure 3. Characterization of transgenic *Arabidopsis* plants with constitutive *ATHB7* expression.(a); Northern blot containing 10 μg of total RNA in each lane, extracted from wild-type control plants (wt) and two independent overexpressor lines of *ATHB7* (S5 and M4). (b)–(f); Wild-type and *35S::ATHB7* transgenic plants grown under non-limiting water conditions. (b); 33-day-old wild-type plant (c); 33-day-old *35S::ATHB7* plant (d); 70-day-old wild-type plant (e); 70-day-old *35S::ATHB7* plant (f); shoot length of wild-type (grey bars) and *35S::ATHB7* transformant (green bars) plants at 28–68 d after germination.

phenotypic deviation from wild type showed a quantitative correlation to the level of *ATHB7* expression, the strongest phenotypic effects being detected in the *35S::ATHB7* S5 line. After initiation of reproductive development the elongation rate of the main inflorescence stem was reduced in these plants and as a consequence, the time point at which the stem reached 5 mm in length was delayed by 8 d in the *35S::ATHB7* plants compared to wild type. The reduction in stem length, caused by the delay in elongation of the inflorescence stem, was maintained throughout the life span of the plants (Fig. 3f). The final length of the main inflorescence stem was reduced by approximately 15% in the *35S::ATHB7* plants in comparison with the wild-type control plants. Plants were also grown under water-limiting conditions. Under these conditions, both the wild-type and the *35S::ATHB7* plants reduced their final inflorescence stem length to the same extent (Fig. 4a), approximately 50%.

In addition to the reduction in inflorescence stem elongation growth, the *35S::ATHB7* plants also showed an increased branching of the main inflorescence stem in comparison with the wild-type plants (Fig. 4b). Plants with elevated levels of *ATHB7* displayed twice the number of side-branch internodal segments, as compared to the wild-type control. Further, rosette leaves of the plants expressing the *35S::ATHB7* transgene were more rounded and petioles shorter as compared to the wild type (Fig. 3b & c). There was no difference in total leaf area, however, between plants expressing the *ATHB7* transgene and wild-type plants (Fig. 4c).

The observed delay in elongation growth of the main inflorescence stem could hypothetically be due either to a delay in the transition to reproductive development or to a delayed onset of elongation of the bolting inflorescence stem. To distinguish between these two alternatives, the

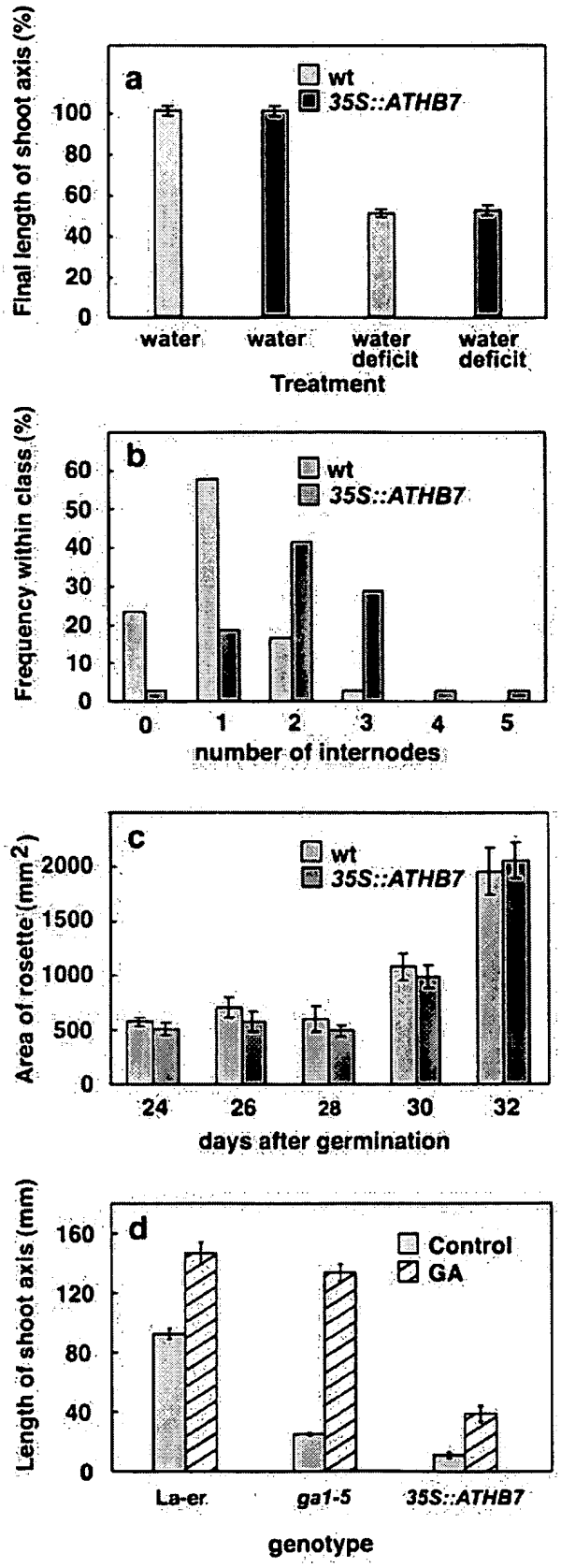


Figure 4. The phenotypic effects of alterations in *ATHB7* expression levels in transgenic *Arabidopsis*. (a) Final length of shoot axis of wild-type (light grey bars) and *35S::ATHB7* transformant plants (dark grey bars) after growth in non-limiting or limiting water conditions, treatments starting at initiation of reproductive development. Measurements were made at 70 d after germination. Lengths of plants grown in limiting water conditions are presented as percentages of the respective lengths in wild-type plants or the *35S::ATHB7* transformant plants grown in non-limiting water conditions (%) ($n = 12$). (b) Final number of side-branch internodes of the main inflorescence stem. The *y*-axis denotes frequency within class of wild-type (light grey bars) and *35S::ATHB7* transformant plants (dark grey bars) (%) ($n = 30$). (c) Total area of rosette leaves of wild-type (light grey bars) and *35S::ATHB7* transformant (dark grey bars) plants at 24–32 d after germination ($n = 6$). (d) Length of shoot axis of Landsberg erecta (*Ler*), the GA biosynthetic mutant *ga1-5* and the *35S::ATHB7* transformant. Light grey bars represent plants sprayed with control solution containing 0.02% Tween-20, and hatched bars represent plants treated with 1 mM GA3 in 0.02% Tween-20. Measurements were performed at 40 d after germination ($n = 12$). In (a), (c) and (d) the results are presented as means, with error bars representing SE ($n = 6$–12).

timing of flower bud initiation was determined in *35S::ATHB7* transformant and wild-type control plants under long day conditions. The result showed that there was no significant difference between the plants in the time point of flower bud initiation under long day conditions. The wild-type plants initiated flower bud formation 15 d after germination (at 6–7 leaves) and the *35S::ATHB7* line 15–16 d after germination (at 6–7 leaves).

To determine whether the difference in inflorescence stem growth between wild-type and *35S::ATHB7* plants was due to a difference in cell numbers along the stem, or to a difference in elongation of the stem cells, we analysed the epidermal cell size of the inflorescence stem in both plant types. Data obtained by use of SEM, demonstrated that the cell number per unit length of stem in plants with a stem length of 100 mm did not differ significantly between plants. The epidermal stem cells of the *35S::ATHB7* and wild-type plants were 321 ± 19 and 299 ± 15 μm, respectively (mean $\pm$ SE, $n = 24$), resulting in no statistical difference (Z-test). Therefore, the reduced final stem length in the *35S::ATHB7* plants is due to reduced stem cell expansion, rather than a reduction in cell numbers in the stem.

During the process of analysing the *35S::ATHB7* plants we noted, in addition to reduced elongation of the inflorescence stem, a slight reduction in the distance between siliques in the *35S::ATHB7* plants compared to wild-type plants. To test whether the ATHB7 effect on stem elongation and the intersilique distance was due to an altered level of, or an altered response to gibberellins, we analysed the effect of GA on inflorescence stem elongation in *35S::ATHB7* plants transformed into the Landsberg *erecta* (*Ler*) background, in the GA-deficient mutant *gal-5* and in wild-type control plants (Fig. 4d). The plants were repeatedly sprayed with either 1 mM GA (GA3) or with a control solution without GA, starting prior to the initiation of reproductive development. The experiment showed that exogenous application of GA did not fully restore the elongation of the transgenic *35S::ATHB7* *Ler* inflorescence stem to wild type and that the *35S::ATHB7* plants respond to GA in a manner similar to the wild-type control. Therefore, the effect of the *35S::ATHB7* transgene on inflorescence stem elongation growth is unlikely to be due to an altered level of active GA or an altered GA responsiveness in the plant.

DISCUSSION

ATHB7 is strongly and rapidly induced by treatments that reduce water availability to the plant, by a mechanism that requires the endogenous production of abscisic acid, as well as the product of the *ABI1* gene (Söderman *et al.* 1996). This data suggests that *ATHB7* constitutes the end-point in a water-deficit signal transduction pathway, which includes the *ABI1* gene product, and that ATHB7 may act as a mediator of a response to reduced water availability (Söderman *et al.* 1996). In this report we demonstrate that constitutive expression of *ATHB7* at elevated levels, in transgenic *Arabidopsis* plants, results in alterations in plant phenotype that are consistent with this hypothesis.

In plants, in which *ATHB7* expression is driven by the constitutive 35S-promoter, the elongation of the main inflorescence stem was significantly reduced, as compared to the wild type. The reduction in stem length, in turn, was the consequence of a reduction in the elongation of the cells of the stem, and was independent of the activity of gibberellins. Similarly, the elongation of the leaves was reduced in the transgenic plants, but the total rosette leaf area was independent of *ATHB7* expression, the primary effect of *ATHB7* expression being an alteration in leaf shape. We conclude from these data that *ATHB7*, when expressed constitutively, acts as a negative regulator of inflorescence stem and leaf cell elongation.

In addition to causing a reduction in final length of the inflorescence stem, *ATHB7* also caused a distinct delay in the onset of its elongation. This delayed bolting was not the result of a delay in the transition from the vegetative to the reproductive growth phase, since the timing of flower induction was unaffected by *ATHB7* expression levels. Instead, *ATHB7* appears to specifically affect the timing of the onset of internode cell elongation in the bolting inflorescence stem. An additional phenotypic change produced by elevated *ATHB7* expression levels was an increased branching of the main inflorescence stem, suggesting a decreased apical dominance in the plants. Even though *ATHB7* transcription is unaffected by exogenous treatments with plant hormones other than ABA (Söderman *et al.* 1996) we can not exclude the possibility that *ATHB7*, when expressed at elevated levels, might affect the response of the plant to other growth regulators, such as auxin or brassinosteroids.

The expression pattern of *ATHB7* in the wild type, as deduced from the pattern of the *ATHB7* promoter activity, is consistent with a function of ATHB7 related to cell elongation, expansion or differentiation in the context of water-deficit stress. The promoter activity was highly induced in expanding inflorescence stems, leaf primordia, siliques and young flower buds after exposure of plants to water limiting conditions. The expression of the gene, thus, is tightly associated with cells that are in an expansion/differentiation phase of growth in both the inflorescence stem and in leaves.

Phenotypic effects of ectopic expression of transgenes in transgenic plants are intrinsically somewhat difficult to interpret, in relation to the wild-type function of the gene, since gene function may differ between cells in which the gene is active in the wild type, and cells where it is not. In the case of *ATHB7*, however, an alteration in expression levels is part of the mechanism by which the activity of the gene is controlled in wild-type *Arabidopsis*, and the level of expression in expanding inflorescence stems and leaves in wild-type plants exposed to limiting water conditions are in the same order of magnitude as that in the transgenic lines. Therefore, we believe that the observed phenotypic effects of elevated expression levels in transgenic *Arabi-*

dopsis on elongation growth, are very likely to reflect the function of the gene in the wild-type plant.

A reduction in inflorescence stem length, as well as a reduction in leaf surface area, and an altered root growth pattern, is part of the response to limiting water conditions in many plant species, including *Arabidopsis*. In our experiments, limiting water conditions in the wild type as well as in the *35S::ATHB7* line, caused a reduction in final length of the stem of about 50%. The transgenic *ATHB7*-expressing plants grown under well-watered conditions also reduced their final length, by approximately 15% compared to wild-type control plants. This data is consistent with ATHB7 being part of a mechanism that regulates inflorescence stem elongation in response to limiting water conditions in the wild-type plant.

The absence of an effect on growth properties from the expression of an antisense construct of *ATHB7* might suggest that a loss of *ATHB7* function in the plant is compensated for by the activity of a second gene, and thus that *ATHB7* gene function might be redundant. Possible candidates for such genes are the HD-Zip genes *ATHB6* (Söderman *et al.* 1999) and *ATHB12* (Lee & Chun 1998). These two genes are also induced by water deficit and ABA, and have expression patterns that partly overlap with that of *ATHB7* (Söderman *et al.* 1999; our unpublished observations). An alternative possibility is that the absence of an effect in these transgenic lines is due to the rather small decrease in steady-state *ATHB7* mRNA levels observed in the transgenic plants. In the absence of data on the phenotypic properties of an *ATHB7* null mutant, we can not distinguish between these possibilities.

Data on the effects of altered expression levels of other HD-Zip class I genes in *Arabidopsis* is limited to a few examples. The effects of ectopic expression of *ATHB1* in tobacco have been interpreted to indicate ATHB1 to be involved in the control of leaf development (Aoyama *et al.* 1995). Constitutive high-level expression of *ATHB13* cDNA in transgenic plants results in altered development of cotyledons and leaves, specifically in plants grown on media containing metabolizable sugars, suggesting ATHB13 to be a component of a sucrose-signalling pathway (Hanson *et al.* 2001). Thus, the functional information available on two genes of the HD-Zip class I implies that these genes mediate the influence of growth conditions on development and in one case control the development of specific cell types. Our data, derived from constitutive expression of *ATHB7*, taken together with the promoter GUS data indicate that ATHB7 may act as a regulator of growth and development of the elongating leaf and the inflorescence stem in response to water availability.

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Evaluation of *Hahb4* performance in Wheat and Maize

Figure 1. Evaluation of wheat (*Triticum aestivum*) GMO lines B and G:

Progeny derived from two independent transgenic events (T_1) were grown and compared with wild-type (non-GMO) germplasm. All individuals were exposed to the same treatment (i.e. fertilization, soil medium, watering, and overall growing conditions), and grain yield was compared for the different genotypes. Watering was discontinued for all individuals once male flowers opened and re-established after severe stress was induced. Progeny expressing Hahb4 show an average 75% increase in yield compared to wild-type individuals subjected to the same condition.

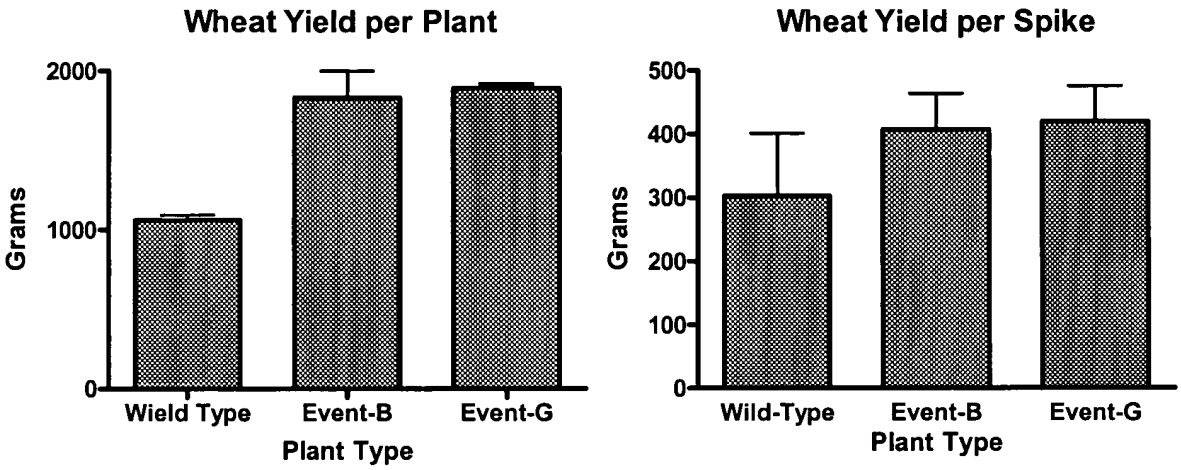

Figure 2. Evaluation of maize (*Zea mays*) GMO lines 3, 6 and 7:

Progeny derived form three independent transgenic events (T_1) were grown and compared with wild-type (non-GMO) germplasm. All individuals were exposed to the same treatment (i.e. fertilization, soil medium, watering, and overall growing conditions), and total leaf surface areas were measured before and after a 4-week water-stress period. Progeny expressing Hahb4 retained on average approximately 50% of total leaf area measured prior to stress induction; whereas the non-GMO control only retained 24% of the original leaf area.